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<b>(21) International Application Number:</b> PCT/GB92/01881 <b>(22) International Filing Date:</b> 14 October 1992 (14.10.92) <b>(71) Applicant:</b> ZENECA LIMITED [GB/GB]; Imperial Chemical House, Millbank, London SW1P 3JF (GB). <b>(72) Inventors:</b> BIRD, Colin, Roger ; 31 Fairfax, Bracknell, Berkshire RG12 1YT (GB). FENTEM, Philip, Anthony ; 9 Aggisters Lane, Wokingham, Berkshire RG11 4DW (GB). KEELING, Peter, Lewis ; 1410 Big Bluestem, Ames, IA 50010 (US). SINGLETARY, George ; 701 Northwest School Street, Ankeny, IA 50021 (US). <b>(74) Agent:</b> HUSKISSON, Frank, Mackie; Imperial Chemical Industries plc, ICI Group Patent dept., P.O. Box No. 6, Bessemer Road, Welwyn Garden City, Herts AL7 1HD (GB).		<b>(81) Designated States:</b> AU, BB, BG, BR, CA, CS, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, PL, RO, RU, SD, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> NOVEL PLANTS AND PROCESSES FOR OBTAINING THEM  <b>(57) Abstract</b>  Plants, particularly cereal plants, which have improved ability to synthesise starch at elevated or lowered temperatures and/or to synthesise starch with an altered fine structure are produced by inserting into the genome of the plant (i) a gene(s) encoding a form of an enzyme of the starch or glycogen biosynthetic pathway, particularly soluble starch synthase and/or branching enzyme and/or glycogen synthase, which display an activity which continues to increase over a temperature range over which the activity would normally be expected to decrease, and/or (ii) a gene(s) encoding sense and anti-sense constructs of enzymes of the starch biosynthetic pathway, particularly soluble starch synthase and/or branching enzyme and/or glycogen synthase, which alters the natural ratios of expression of the said enzymes or inserts enzymes with special structural characteristics which alter the natural branching pattern in starch.		

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## NOVEL PLANTS AND PROCESSES FOR OBTAINING THEM

This invention relates to novel plants having an improved ability to produce starch including an improved ability to produce structurally-altered starch. Such novel plants are capable of producing higher yields than known plants, and/or are capable of producing starch of altered quality. The invention further relates to processes for obtaining such plants.

Temperature is one of the most important ecological factors governing the natural distribution of plants and their satisfactory growth and yield potential in agricultural cultivation. A crop will give its highest yields, and lowest risk of failure, when it is cultivated as close as possible to the specific temperature optima for each of its development stages in the course of the growing season. In many agricultural regions, temperature is not a stable factor for crops with extended growth periods and the plants may suffer stress because of temperatures which are too high or too low, or both, in different intensities and over short or long time intervals.

Many of the world's food crops are cultivated in regions where their yield is constrained by what we have called "thermal thresholds" for optimal growth (Keeling and Greaves, 1990). The actual optimum temperature for maximal yield differs amongst different crops: for example, it is well known that certain cereal plants (such as wheat, barley and maize) give a maximum grain yield at around 25° to 30°C. This optimum temperature is the basis for the calculation of Heat Units (HU)

which are used to calculate Growing Degree Units (GDU). GDU is a measure of the HUs a plant requires within a particular temperature range to reach a maximum yield.

5           "Grain filling" is dry matter accumulation in the grain, and occurs over the period during which the grain increases in weight. Published information on crops such as wheat, barley, maize, rice and sorghum, shows that as the growth  
10 temperature increases the duration of grain filling declines. At temperatures below the optimum temperature, the rate of grain filling increases with increasing temperature. This compensates or even overcompensates for reduced duration of grain  
15 filling such that, overall, the yield increases with temperature (Figure 1). However, at higher temperatures the grain filling rate fails to increase further with temperature and, indeed, declines with temperature increases above 30°C.  
20 These changes in grain filling rate fail to compensate for further reduction in grain filling duration. The overall effect is reduced yield (and also starch quality) because cereal crops spend significant proportions of time during the grain  
25 filling period at temperatures which are higher than optimal for grain filling rate. Thus the limitations on grain filling rate imposes a penalty on the total amount of dry matter that can be accumulated in any one growing season. In addition  
30 there is a change in starch fine structure, affecting its quality, because the amylose/amylopectin ratio is affected and the starch granule density is reduced. Although these effects are documented in the literature, their

cause has hitherto been unknown.

Arrhenius first developed a generalisation for the effects of temperature on the rates of biochemical reactions and proposed a unifying mechanism involving energy of activation ( $E_a$ ) of chemical reactions. This has become known as the Arrhenius Equation:

$\ln(k_2/k_1) = (E_a/R)(1/T_1 + 1/T_2)$  where the  $k$  values represent the velocity constants of each temperature ( $T$ ) and  $R$  is the gas constant. The Arrhenius equation defines a chemical mechanism for the generalisation of a factor " $Q_{10}$ " where the rate will double or treble for a  $10^\circ\text{C}$  rise in temperature.

$$Q_{10} = \text{Velocity } (T + 10)^\circ\text{C} / \text{Velocity } T^\circ\text{C}$$

Enzymes, which determine the velocity of any chemical reaction in living organisms, act by lowering the free energy of activation values of the chemical reactions to the extent that the thermal energy which is present in the organism is sufficient to activate the reactants. Thus the  $Q_{10}$  values associated with enzyme catalysed reactions are therefore a physical characteristic of the energy of activation achieved by that enzyme.  $Q_{10}$  values will therefore remain fixed provided (i) the catalytic site of the enzyme-protein remains functional and (ii) the substrate itself is not affected significantly by changed temperature. It is a well known fact that at temperatures which exceed the thermodynamic stability of proteins (normally considered to be about  $40$  to  $50^\circ\text{C}$ ) the protein structure will "unfold" and its catalytic function will become disrupted and hence it will no longer favour conversion of substrates to products.

This phenomenon is well characterised in the literature and has been extensively studied in a variety of living systems as well as in thermophilic organisms. The finding that dry  
5 matter accumulation in starch-storing crops apparently has an optimum temperature of 25 to 30°C is very unusual because this temperature is lower than the temperature at which proteins are normally considered to become disrupted. The phenomenon  
10 thus lacks any explanation in the literature.

Our previous studies have led to a new understanding of the metabolic pathway of starch synthesis in developing starch storing tissues (Keeling et al, 1988, Plant Physiology,  
15 87:311-319; Keeling, 1989, ed. C.D. Boyer, J.C. Shannon and R.C. Harrison; pp.63-78, being a presentation at the 4th Annual Penn State Symposium in Plant Physiology).

We have established that grain filling rate  
20 and duration is governed by factors in the grain itself rather than being due to some component of the source tissues. Since starch comprises up to 75% of the grain dry weight in cereals it is logical that the limitation to plant dry matter  
25 accumulation referred to above will be most likely due to an effect on starch deposition. This suggests that cereal species or varieties with higher yield will be characterised by either longer duration of starch synthesis in the grain or  
30 greater increases in the rate of starch synthesis at elevated temperatures.

Furthermore, this work has given us an understanding of the factors involved in determining the optimum growth temperatures of all

starch storing plants, and an understanding of the enzymes responsible for determining the fine structure of starch. In particular, we have investigated the biochemical reactions and  
5 interactions between the soluble starch synthase and branching enzymes which contribute to the fine-structure of starch deposition in plants.

An object of the present invention is to provide novel plants having an increased capacity  
10 to produce starch and a capacity to produce starch with an altered fine structure.

According to the present invention there is provided a method of producing a plant with altered starch synthesising ability comprising  
15 stably incorporating into the genome of a recipient plant one or more than one donor gene specifying an enzyme involved in a starch or glycogen biosynthetic pathway.

The above method is generally applicable to  
20 all plants producing or storing starch. The recipient plant may be: a cereal such as maize (corn), wheat, rice, sorghum or barley; a fruit-producing species such as banana, apple, tomato or pear; a root crop such as cassava,  
25 potato, yam or turnip; an oilseed crop such as rapeseed, sunflower, oil palm, coconut, linseed or groundnut; a meal crop such as soya, bean or pea; or any other suitable species. Preferably the recipient plant is of the family Gramineae and most  
30 preferably of the species Zea mays.

The method according to the invention may be used to produce a plant having an improved capacity to produce starch at elevated or lowered temperature. As noted previously, yield increases

with temperature until a temperature optimum is reached. Above the temperature optimum, yield begins to decrease. Thus plants growing above (or below) their temperature optimum may not be reaching their full yield potential. Improving the plant's capacity to produce starch at temperatures above or below the temperature optimum will result in increased yield. This may be achieved by increasing the amount or type of starch synthesising enzymes present in the plant. It may also be achieved by altering the actual temperature optimum of starch synthesis to suit the growing conditions of a particular plant. Thus a crop variety may be produced which is adapted to the growth temperature of a particular environment (including particular sites or geographical regions). Normally, it is most useful to improve starch production at temperatures in excess of the normal optimum temperature.

The method according to the invention may also be used to produce a plant having the ability to synthesise starch with an altered fine structure. This may be due to a shift in the temperature optimum of starch synthesis or due to other her reasons (such as a change in the overall balance of the different enzymes in the biosynthetic pathway). The fine structure of the starch affects its quality. It is thus possible to generate crops producing starch which is better adapted or targetted to the crops' end-use (such as starch with improved processing properties, improved digestibility, etc).

The method and resulting alterations in starch synthesising ability are particularly advantageous



for maize. The temperature optimum of starch synthesis pathway enzyme activity may be matched more closely to the higher temperature ranges encountered in the typical maize growing regions of the world. In addition, the fine structure of the starch may be changed so that novel starches are made in the recipient plant.

The donor gene to be used may be obtained from any biological source which produces starch or glycogen-synthesising enzymes: it may be of plant origin or fungal origin or bacterial origin or animal origin. For example, donor genes may be derived from a plant selected from the following species Zea mays (especially the varieties Lima 38, Guanajuato 13, Lima 45, Doebley 479 or teosinte 154), Zea diploperennis, Zea luxurians, Zea perennis, Zea tripsacum, Zea parviglumis, and Zea mexicana.

Preferably the donor gene specifies soluble starch synthase (E.C. 2.4.1.21) and/or branching enzyme (E.C. 2.4.1.18) and/or glycogen synthase of bacterial origin (E.C. 2.4.1.21) or animal origin (E.C. 2.4.1.11).

We describe the isolation, purification and characterisation of the enzymes soluble starch synthase and branching enzyme and the production of antibodies which can be used in the identification of soluble starch synthase and branching enzyme cDNA clones. We also describe the use of cDNA clones of plant soluble starch synthases (SSS - soluble starch synthase), plant and bacterial branching enzymes (BE - branching enzyme) and plant and animal and bacterial glycogen synthases (GS - glycogen synthase).

The donor gene may be an additional copy of the gene specifying the normal enzyme present in the plant (that is, an additional copy of the wild-type gene). Increased gene expression may  
5 also be elicited by introducing multiple copies of enhancer sequences into the 5'-untranscribed region of the donor gene.

The donor gene may specify an alternative enzyme with improved properties compared to those  
10 of the normal plant enzyme. For example, glycogen synthase (involved in the glycogen biosynthetic pathway) may be seen as equivalent to soluble starch synthase (in the starch biosynthetic pathway) as it catalyses a similar reaction.  
15 However, glycogen synthase (such as glgA found in E coli) has a higher temperature optimum of activity than soluble starch synthase, and so its expression in the plant should improve the plant's capacity to produce starch at elevated temperatures. A second  
20 example is bacterial branching enzyme (such as glgB from E coli) which is equivalent to plant branching enzyme, but may have improved properties.

The donor gene may also specify a modified allelic form of the enzyme with kinetic or  
25 allosteric properties different to those of the normal plant enzyme. In particular, the enzyme encoded by the donor gene may have a temperature optimum of activity higher than that of the recipient plant enzyme, or may show enhanced  
30 thermal-stability (which may enhance the duration of grain filling).

Expression of improved allelic forms of an enzyme within a recipient plant will produce a plant with increased starch-yielding capacity

and/or better starch quality. Examples of such plants include: a plant (especially maize) having a starch synthesising ability which does not decrease with temperature between 25 to 30°C; a plant  
5 (especially maize) containing a soluble starch synthase and/or branching and/or glycogen synthase enzyme with a  $Q_{10}$  value greater than 0.8 between 25 and 35°C; a plant (especially maize) containing a  
10 soluble starch synthase and/or branching and/or glycogen synthase enzyme which is resistant to reduction of activity after exposure to a temperature in excess of 40°C for two hours.

Genes encoding improved allelic forms may be obtained from suitable biological organisms, or  
15 endogenous wild-type genes may be manipulated by standard protein or genetic engineering techniques.

Starch-producing and glycogen-producing organisms (including plants, fungi, bacteria, animal cells) may be used as sources of improved  
20 enzyme genes. Such organisms may be screened for allelic forms of the enzyme which are more catalytically active than those typically found in the crop. It is also possible to alter the properties of the enzyme in the recipient plant  
25 through protein and genetic engineering. Genes encoding variants of the enzymes may be created using molecular techniques or mutagenesis.

The donor gene may be an antisense sequence which reduces expression of the enzyme in the  
30 recipient plant. For example, this may be used to alter the balance of the different starch-synthesising enzymes present in the cell which may change the amount or type of starch produced.

In particular, it is possible to insert genes in both sense and/or anti-sense orientations in order to effect a change in the natural ratios of various isoforms of branching enzyme and soluble starch synthase in the recipient plant. Altering the natural ratios of the soluble starch synthase and branching enzyme activities of the recipient plant results in starch being produced with new and novel branching structures, amylose/amylopectin ratios, and an altered starch fine structure.

An antisense donor gene (used to reduce expression of the wild-type gene) may also be incorporated with a sense donor gene encoding a more active replacement enzyme. For example, a sense gene encoding glycogen synthase may be incorporated together with an antisense gene for soluble starch synthase to potentiate the effect of increased temperature optimum described above.

It is possible to insert more than one copy of the donor gene into the recipient genome. Each donor gene may be identical, or a combination of different donor genes may be incorporated. For example, the donor genes may have differing sequences which may encode more than one allelic form of the enzyme or may be derived from more than one source.

In summary, the following are examples of genetic manipulation methods which may be used to produce plants with an altered ability to synthesise starch:

- (1) Insertion of an additional copy of the wild-type enzyme gene;
- (2) Insertion of a more active or more thermally resistant enzyme gene obtained from a

suitable biological organism;

(3) Insertion of multiple copies of the wild-type or enhanced activity enzyme gene;

(4) Modification of the enzyme gene by techniques known in protein engineering to achieve alterations in the kinetics and/or allosterics of the enzyme reaction (eg to achieve thermal stability);

(5) Modification of the promoter sequences using techniques known in protein engineering to achieve enzyme over-expression;

(6) Modification of the sequence of the enzyme gene and/or its promoter and/or the transit peptide.

The said donor gene may be derived from a sexually compatible donor plant and inserted into the recipient plant by sexual crossing of donor and recipient plants.

Alternatively, the donor gene may be isolated from a suitable biological organism, such as a plant, bacterium, fungus or animal cell. Insertion of the donor gene is effected by genetic transformation of the recipient plant. If the donor organism is not sexually compatible with the recipient, the gene to be incorporated into the recipient plant genome is excised from the donor organism and the genome of the recipient plant is transformed therewith using known molecular techniques. The advantages of the transformation method are that isolated enzyme genes, genes from diverse biological sources and/or anti-sense constructs (not just sense constructs) may be incorporated in the recipient plant genome.

Preferably the recipient plant is of the

family Gramineae and most preferably of the species Zea mays. Other recipient plants may include rice, wheat, or barley, and fruit crops such as tomato.

5 The invention also provides a plant having one or more than one donor gene specifying an enzyme involved in a starch or glycogen biosynthetic pathway stably incorporated into its genome such that its ability to produce starch is altered. Such plants may have an improved capacity to  
10 produce starch at elevated or lowered temperature, and/or an ability to synthesise starch with an altered fine structure. Hence such plants are capable of producing higher starch yields at certain temperatures and/or are capable of  
15 producing starch with an improved quality.

The variety of possible plants, donor genes and resulting effects have been discussed previously.

20 The invention also provides the seeds and progeny of such plants, and hybrids whose pedigree includes such plants.

The present invention will now be described, by way of illustration, by the following description and examples with reference to the  
25 accompanying drawings of which:

Figure 1 is a graph showing the effect of temperature on final grain weight.

Figure 2 is a graph of starch synthesis rate against temperature.

30 Figure 3 is a graph of field temperature against time spent above that temperature.

Figure 4 shows computer simulations of different rate-models of temperature/activity profiles.

Figure 5 is a graph of rate against temperature for several starch-synthesising enzymes.

5 Figure 6 is a graph of  $Q_{10}$  values against temperature for several starch-synthesising enzymes.

Figure 7 is a graph of rate against temperature for maize endosperm soluble starch synthase and branching enzyme.

10 Figure 8 is a graph of  $Q_{10}$  values against temperature for maize endosperm soluble starch synthase and branching enzyme.

Figure 9 is a graph of rate against temperature for rice, maize, sorghum and millet soluble starch synthase.

Figure 10 is a graph of  $Q_{10}$  values against temperature for rice, maize, sorghum and millet soluble starch synthase.

20 Figure 11 is a graph showing soluble starch synthase and ADPG pyrophosphorylase activity against pre-incubation temperature.

Figure 12 is a graph showing soluble starch synthase activity and starch synthesis against pre-incubation temperature.

25 Figure 13 is a graph showing soluble starch synthase activity against time after pre-incubation at 40°C.

30 Figure 14 is a graph showing recovery of soluble starch synthase and UDPG pyrophosphorylase activity against time after heat treatment.

Figure 15 is a graph showing  $Q_{10}$  values of soluble starch synthase and glycogen synthase against temperature.

Figure 16 is a graph of soluble starch

synthase activity against temperature using various primers.

Figure 17 is a graph of soluble starch synthase activity against temperature using various primers.

Figure 18 shows the nucleotide sequence for E coli glycogen synthase.

Figure 19 shows the construction of two glycogen synthase transformation vectors.

Figure 20 is a graph showing the frequency distribution for  $Q_{10}$  values (25 to 35°C) for different maize lines.

Figure 21 is a graph showing soluble starch synthase activity against temperature for various maize genotypes.

Figure 22 is a graph showing soluble starch synthase activity eluted from the mono-Q column.

Figure 23 shows activity of various starch synthesising enzymes at different gene doses in the sugary maize mutant.

Figure 24 shows activity of various starch synthesising enzymes at different gene doses in the dull maize mutant.

Figure 25 shows activity of various starch synthesising enzymes at different gene doses in the shrunken maize mutant.

Figure 26 shows activity of various starch synthesising enzymes at different gene doses in the amylose extender maize mutant.

#### EXPERIMENTAL BACKGROUND TO THE INVENTION

In order to examine in detail the relationship between starch synthesis rate and temperature in vivo, we have conducted experiments on maize plants



growing under defined temperature regimes in constant environment cabinets. In these experiments the maize ears have been maintained at defined temperatures independently of the rest of the plant by means of thermostatically controlled glass fibre heating mantles placed around the ears. Rates of starch synthesis at precisely regulated temperatures were calculated from dry weight accumulation curves for the maize kernels over a defined period. The analysis of maize endosperm starch synthesis at different temperatures showed that the rate increases with temperature between 15 and 25°C. Between 25 and 30°C there was no significant increase in rate. The rate at 35°C was significantly lower than the rate at 30°C (Figure 2).

To examine the relevance of the in vivo starch accumulation rate versus temperature curve in the field, we monitored field temperatures during the starch filling period at various field stations in the mid-west of the United States. Air temperatures were monitored using a thermocouple attached to a recording device which recorded temperatures at five minute intervals. In addition, temperatures within the maize kernels were recorded by inserting thermocouples into the grain. Significant periods of time are spent above the optimal temperature (Figure 3). We used computer modelling techniques to calculate the expected yield benefits of altering the in vivo starch synthesis versus temperature curves in several defined ways (Figure 4). For example, increasing the temperature optimum for grain filling rate from 25 to 30°C gives an anticipated

yield benefit in excess of 10% over the years which we have recorded (Table 1).

TABLE 1

5 Computed increases in yield for a 5°C  
increase in temperature optimum

Location	Year	Real Rate Model	
		Air	Cob
Cisco, Illinois	1986	11.4%	-
Cisco, Illinois	1987	15.9%	-
Slater(1) Iowa	1988	-	15.7% ±1.2
Slater(2) Iowa	1988	15.9%	15.9% ±0.2
Cottage Grove Wisconsin	1988	20.4%	24.1% ±1.1
Wood River Nebraska	1988	15.8%	15.7% ±0.7

In addition to in vivo information on temperature dependence, we have obtained in vitro evidence on the biochemical basis for the temperature dependence. We applied  $^{14}\text{C}$ -glucose to developing maize grain in vitro at different temperatures and measured the flux of radioactivity through different pathways. Using  $1\text{-}^{14}\text{C}$ -glucose and  $6\text{-}^{14}\text{C}$ -glucose it was possible to determine the in vitro rates of glycolysis and the pentose phosphate pathway as well as starch synthesis. The data in Table 2 below show that between 20 and 40°C there was no significant increase in the rate of starch synthesis, though the flux of radioactivity through the pentose phosphate pathway and glycolysis increased approximately three-fold and two-fold respectively. The failure of the starch synthesis rate to increase above 25°C is therefore due to the starch synthesis enzymes in the amyloplast (i.e. any one or any combination of soluble and bound starch synthase, branching enzyme and ADP-glucose pyrophosphorylase), and not due to a failure in the supply of sucrose or ATP at high temperatures.

TABLE 2

Effect of temperature on radioactivity  
released as carbon dioxide or incorporated  
into starch from 1-14C and 6-14C glucose

	Temperature	
	20°C	40°C
Krebs Cycle mmol CO <sub>2</sub> /hr/endosperm	14.8 ±6	33.4 ±8
Pentose P. pathway mmol CO <sub>2</sub> /hr/endosperm	3.8 ±3	11.3 ±3
Starch mmol glucose/hr/endosperm	45.7 ±4	54.9 ±7

25 To analyse further the biochemical basis for  
the temperature optimum for grain filling rate in  
cereals, we measured the activities with respect to  
temperature of most of the maize endosperm enzymes  
in the pathway for converting sucrose to starch,  
including soluble starch synthase (SSS) and  
branching enzyme (BE). Two aspects have been  
30 studied:

(1) The temperature dependence of the rates of the  
reactions catalysed by these enzymes; and,

(2) The stabilities of the individual enzymes during incubations at different temperatures both in vivo and in vitro.

5 The glycogen synthase enzyme has also been studied (see (3) below).

(1) Temperature Dependence

10 The reaction rates of most of the enzymes studied (alkaline pyrophosphatase, phospho-  
glucomutase, UDP glucose pyrophosphorylase, hexokinase, phosphoglucosylase, sucrose  
synthase, ADP glucose pyrophosphorylase and bound  
starch synthase) have activities which increase  
with temperature at least up to 45°C (Figure 5).  
15 This temperature exceeds the highest temperature recorded during grain filling in the field. When  
replotted as  $Q_{10}$  values (Figure 6) it is clear that these enzymic reactions are stable across this  
temperature range. This is consistent with the  
20 theoretical expectation from the Arrhenius equation referred to above.

The temperature responses of SSS and BE were, however, a complete contrast (Figure 7). The  
apparent temperature optima for activity are 25°C  
25 for SSS and 27.5°C for BE. When replotted as  $Q_{10}$  values (Figure 8) it is clear that there is an  
apparently constant decline in enzymic efficiency with increasing temperature. This is indicative of  
some decrease in catalytic activation brought about  
30 by depressed interaction between the enzyme and its substrates. As these are the only enzymes in the  
pathway with temperature optima for activity below 30°C, it is apparent that one or both of SSS and BE  
are "rate limiting" for starch synthesis between 20

and 30°C. The failure of starch synthesis rate in vivo to increase with temperature between 25 and 35°C must be due to a failure of the activities of one or both of these enzymes to increase in activity over this temperature range.

Grain has been sampled from maize plants grown at 20, 25 and 30°C and SSS and BE activity has been measured over several temperatures. The temperature at which the grain are growing was found to have no effect on the temperature optima for activity of these two enzymes. Temperature activity curves for these two enzymes from the endosperms of several commercial U.S. maize hybrids show the same temperature optima. As shown in Figure 8,  $Q_{10}$  values for in vivo rates and SSS and BE activities show remarkable similarities. This is in contrast to, for example, ADP-glucose pyrophosphorylase where  $Q_{10}$  is fixed across different temperatures as predicted by the Arrhenius equation.

Comparisons of the temperature activity curves for SSS from maize, rice, sorghum and millet show that the temperature optima for activity were consistently around 25°C (Figure 9). There were, however, differences in  $Q_{10}$  between 15 and 25°C, the  $Q_{10}$  increasing in the order maize, sorghum, rice and millet (Figure 10), that is, in the order of increasing climatic temperatures of the locations where the crops are grown. Further studies of dicot plants has led to the discovery that the  $Q_{10}$  for SSS can be considerably higher in some plants than we have observed in the monocot plants so far studied. For example in tomato leaf and fruit the  $Q_{10}$  values were

significantly higher than that seen in maize (Figure 9).

5 High increases in  $Q_{10}$  between 15 and 25°C mean that SSS may not become rate limiting at 25°C, pathway rate control being passed to another enzyme(s). The in vivo rate of flux through the starch pathway may, therefore, continue to increase with temperature above 25°C.

10 The temperature activity curves of the green leaf tissue forms of SSS and BE are similar to those observed for the developing grain. These enzymes, we conclude, limit photosynthesis at temperatures above 25°C.

15 The temperature activity curves of the developing tassel forms of SSS and BE are similar to those observed for the developing grain forms, again confirming that these two enzymes limit starch synthesis at temperatures above about 25°C.

## 20 (2) Temperature Stability

Experiments with both wheat and maize have confirmed that increased temperature not only affects the rate of enzyme catalysed reaction of SSS, but also results in enzyme instability  
25 resulting in long term loss of enzyme activity. We have termed this phenomenon "knockdown" in order to distinguish it from the changes in reaction rate described above. "Knockdown" is defined as the change in maximum catalytic activity assayed at  
30 20°C following a heat treatment of the enzyme either in vitro or in vivo at a higher temperature.

Incubation of extracted maize endosperm SSS in vitro at temperatures up to 37°C did not cause any loss of enzyme activity when subsequently assayed

at 20°C. At temperatures higher than 37°C, however, progressive long term loss of enzyme activity occurred, such that by 40°C, 80% of the enzyme had become inactivated. When peeled maize grains were preincubated for three hours at 40°C, there was a significant drop in SSS activity when subsequently extracted and assayed at 20°C (Figure 11). In contrast the extractable activity of ADP-glucose pyrophosphorylase was not altered by this same preincubation. Pre-treatments for three hours at 10, 20 or 30°C did not result in any drop in the activity of SSS. Similar in vitro incubations of wheat grain resulted in substantial loss of SSS activity at temperatures as low as 30°C while ADP-glucose pyrophosphorylase activity was unaffected. The drop in SSS activity at 40°C coincided with a drop in starch synthesis measured as <sup>14</sup>C incorporation into starch assayed in-vitro (Figure 12).

In experiments in which maize ears were heated in situ on the plant, temperatures in excess of 37°C caused loss of SSS activity when subsequently extracted and assayed at 20°C. The activity of ADP-glucose pyrophosphorylase was, again, unaffected by this treatment. Following heat treatments at 40°C for a range of times, it was found that the extractable SSS activity declined dramatically over a two hour period (Figure 13). When the cobs were returned to 20°C following a period at 40°C, the extractable SSS activity only returned to its initial activity after a period of 24 hours at the lower temperature (Figure 14). Similar experiments with wheat ears also showed that knockdown of SSS occurred in vivo



at temperatures of 30°C and above. Recovery of SSS activity in wheat also took over 24 hours.

(3) Temperature Dependence of Glycogen Synthase

5           The biochemical reaction catalysed by soluble  
starch synthase (ADPGlucose:1,4- $\alpha$ -D-glucan4- $\alpha$ -D-  
glucosyltransferase (E.C. 2.4.1.21)) involves the  
sequential addition of glucose donated from the  
sugar nucleotide ADPGlucose to a glucan chain using  
10       an  $\alpha$ -1,4 linkage. Other sources of this chemical  
reaction exist in nature as the enzymes glycogen  
synthase notable from bacteria (ADPGlucose:  
1,4- $\alpha$ -D-glucan4- $\alpha$ -D-glucosyltransferase  
(EC2.4.1.21)) and animals (UDPGlucose:glycogen  
15       4- $\alpha$ -D-glucosyltransferase (EC2.4.1.11)).

          When we studied the temperature dependence of  
the glycogen synthase reaction we found that there  
was a remarkable increase in activity with  
increasing temperature. This was in dramatic  
20       contrast to the temperature dependence of the  
reaction catalysed by soluble starch synthase  
(Figure 15). The biochemical cause of this new  
temperature response of glycogen synthase indicates  
that this enzyme can maintain its normal activation  
25       energy for the reaction even at higher temperatures  
that reduce SSS activity. The glycogen synthase  
enzymes catalytic site is either able to tolerate  
the higher temperature or else is able to  
compensate for possible changes in the conformation  
30       of the substrate itself. Thus because glycogen  
synthase catalyses the same chemical reaction as  
SSS, it has very wide application as a means of  
increasing plant starch deposition at higher  
temperatures.

The nucleotide and amino-acid sequences of glycogen synthase are known (i) from E.Coli GenBank/EMBL #J02616 (Kumar et al, J Biol Chem 34 16256-16259 (1986)), (ii) from rabbit skeletal muscle (Hang et al, FASEB J 3 3532-3536 (1989)), and (iii) from human muscle (Browner et al, Proc Nat Acad Sci 86 1443-1447 (1989)). The bacterial (ADPGlucose: 1,4- $\alpha$ -D-glucan-4- $\alpha$ -D-glucosyl-transferase (EC 2.4.1.21)) and animal (UDPGlucose: glycogen 4- $\alpha$ -D-glucosyltransferase (EC 2.4.1.11)) sequences are NOT homologous. Furthermore the bacterial forms are not phosphorylated and also are not allosterically affected by glucose 6-phosphate. Finally, the bacterial enzyme uses ADPG (ie, like plants) and only the animal forms use UDPG. This makes the bacterial enzyme the ideal choice for using on plants. The structural genes for the bacterial glycogen synthase are mapped to pop12 in E.Coli and glycogen synthase map to glgA.

Nucleotide sequencing further refines the position of glgA. The translation start of glgA is known to be immediately after glgC and the nucleotide sequence determined. The NH<sub>2</sub> sequence was known so that the actual start of the glgA gene was unambiguously determined as well as confirming the direction of transcription. The deduced amino acid sequence show complete homology with the known NH<sub>2</sub> sequence and with the known amino acid sequence. Different bacterial enzymes show 90% sequence homology. There is complete agreement between the reported and deduced amino acid sequences for the enzyme. Cells transformed with the gene produce a polypeptide that has sequence homology with the known amino acid sequences.

THE BIOCHEMICAL CAUSE OF THE TEMPERATURE  
EFFECT

As was stated above, the  $Q_{10}$  characteristics of an enzyme catalysed reaction is determined by the energy of activation of that enzyme. The atypical behaviour of SSS and BE to increasing temperature shows that the normal activation energy of this reaction which appears to be optimal at lower temperatures (for example, around 10°C) is not carried through to the higher temperatures. The decay in enzyme rate with increasing temperature is indicative of some change in the interaction between the enzyme and its substrate. This could be due to a change in the enzyme catalytic site or else a change in the conformation of the substrate itself. Studies of the effects of different primers on the temperature-dependence of SSS activity shows that the precise temperature optimum is influenced by the nature of the substrate (Figures 16 and 17). Furthermore, when very low molecular weight primers were used in the enzyme reaction the temperature response curve was dramatically altered such that the  $Q_{10}$  was 1.6 between 25 and 35°C for maltotriose, maltotetrose and maltoheptose primers. Collectively these data indicate that the biochemical cause of the temperature optimum of SSS activity is related in some way to the molecular structure of starch and its interaction with SSS and BE.

30

STARCH FINE STRUCTURE ALTERATIONS

Detailed studies of the starch mutants in corn (amylose extender, sugary, dull and waxy) has shown-us that the expression of enzyme activities

is altered in these mutants such that there are new ratios of several enzymes involved in the pathway of starch synthesis. Figures 23 to 25 show the considerable over-expression of several enzymes in the pathway of starch synthesis, particularly where there is a specific lesion in the normal pattern of starch deposition. Furthermore we have found that not only is there a change in overall enzyme expression of some enzymes, but also there is an over-expression of some isoforms of SSS and BE enzymes. These mutations therefore not only cause a reduction in expression of SSS and BE enzymes, as has been reported in the literature, but there is apparently an over-expression of the other isoforms of these same enzymes in the pathway. This change in enzyme isoform expression occurs in response to or as a consequence of a change in flux of sugars to starch. There is then a resulting change in starch fine structure which has been documented in the literature, which is now known to be due to a combination of a reduction in expression of SSS and BE, together with an over-expression of other isoforms of SSS and BE which have different roles in the starch assembly process. These new findings from our ongoing work has led to our efforts to alter gene-expression levels of SSS and BE in cereal endosperm in order to effect a new starch structure in corn. Furthermore, since we now know that different isoforms have different roles in starch assembly there is the opportunity of transforming corn with forms of SSS and BE that have other different properties which will effect a change in the fine structure of the starch (eg using SSS and BE from other diverse biological

sources of plants, animals, bacteria and fungi).

#### CONCLUSIONS FROM THE EXPERIMENTS

Our results have demonstrated that the failure  
5 of the starch synthesis rate in cereal endosperm to  
increase with temperature above 20-25°C is due to  
the fact that in the temperature range of 25-30°C  
the rate of starch synthesis is controlled by the  
activities of the SSS and BE enzymes. The rates of  
10 the reaction catalysed by SSS and BE fail to  
increase with temperature above 25°C. In addition,  
knockdown of SSS activity may further reduce yields  
in maize above 37°C and in wheat above 30°C. Crops  
such as wheat, barley and maize frequently  
15 experience temperatures above 25°C during their  
grain filling period. This invention increases the  
temperature optimum for the grain filling rate  
above 25°C by relieving the limitations imposed by  
the reaction properties of SSS, BE or both. The  
20 enzyme glycogen synthase has a radically different  
response to temperature and is an ideal source of  
enzyme for increasing starch synthesis at  
temperatures that exceed the temperature optima of  
SSS and or BE. Changing the temperature optimum  
25 for starch synthesis in plants increases plant  
yield as well as changing other important  
properties such as fruit texture and sweetness.

Furthermore the alterations in expression  
levels of SSS and BE using both sense and antisense  
30 constructs results in an alterations in the fine-  
structure of the starch produced in the recipient  
plants. In addition, effecting this change in  
ratios of enzyme expression using temperature  
stable enzymes results in a more stable and

defined type of starch quality that is unique to the recipient plants.

5

#### USE OF GLYCOGEN SYNTHASE

A gene encoding a glycogen synthase (GS) enzyme may be extracted from bacteria or animals and introduced into a donor plant by transformation.

10

The temperature sensitivity of starch synthesis may be improved by transforming plant genomes with a gene encoding glycogen synthase. Referring to Figure 15 herewith, it can be seen that the activity of the glycogen synthase enzyme continues to increase with temperature at least to around 40°C, well in excess of the temperature maxima of the other plant enzymes, soluble starch synthase and branching enzyme, associated with starch synthesis. This data confirms the status of glycogen synthase as an enzyme with high Q10. It is also mentioned that the glycogen synthase temperature stability is better than any of the corn-derived enzymes from even the best of the germplasm which has been screened for this property.

25

Glycogen synthase catalyses the same reaction as soluble starch synthase [ADPglucose:1,4- $\alpha$ -D-glucan-4- $\alpha$ -D-glucosyltransferase (E.C. 2.4.1.21)] which catalyses the sequential addition of glucose donated from the sugar nucleotide ADP-glucose to a glucan chain using an  $\alpha$ -1,4 linkage. Other sources of this  $\alpha$ -1,4 linkage reaction exist in nature as the enzyme glycogen synthase, notably from bacteria [ADPglucose: 1,4- $\alpha$ -D-glucan 4- $\alpha$ -D-glucosyl-

30

transferase (E.C. 2.4.1.21)) and animals  
[UDP-glucose: glycogen 4- $\alpha$ -D-glucosyltransferase  
(E.C. 2.4.1.11)]. Again the reaction involved is  
the sequential addition of glucose donated from a  
5 nucleotide sugar to a glucan chain via an  $\alpha$ -1,4  
linkage.

While not wishing to be bound by this  
particular explanation, it is believed that the  
different stability to increased temperature  
10 between soluble starch synthase and glycogen  
synthase is connected with changes in the structure  
of glucan chains at higher temperatures. It is  
generally agreed that the structure of starch (at  
least in solution) is a "statistical helix", that  
15 is, it exists as a dynamic mixture of structured  
helices and random coils. At temperatures much in  
excess of 20-25°C, the hydrogen bonding between the  
glucan double helices begins to break down,  
favouring the less organised random coil  
20 conformation. It is hypothesised that the active  
site of soluble starch synthase lies on the glucan  
chain when present as a helix whereas the glycogen  
synthase site of action is found on the random coil  
structure. By this reasoning, their enzymatic  
25 reaction may be the same but their temperature  
responses dramatically different.

The most favoured sources of the glycogen  
synthase gene for use in this invention are  
bacterial rather than animal sources for the  
30 following reasons:

- (1) the bacterial glycogen synthase and plant  
soluble starch synthase both use ADPG, whereas the  
animal GS enzyme uses UDPG;
- (2) the bacterial GS and plant SSS enzymes do not

have any phosphorylation sites for activation,  
whereas the animal enzyme does; and,  
(3) the animal GS enzyme requires  
glucose-6-phosphate as a co-factor and is  
5 allosterically activated, whereas the plant SSS and  
bacterial GS enzymes are not.

For these reasons the bacterial GS gene is  
preferred. The nucleotide and amino acid sequences  
are known from the literature, for example, E.coli  
10 GenBank/EMBL #J02616 (Kumar et.al., J.Biol.Chem.  
34, 16256-16259 (1986); rabbit skeletal muscle  
(Zhang et.al., FASEB J. 3, 2532-2536 (1989); and,  
human muscle (Browner et.al., Proc,Natl.Acad.Sc.  
86, 1443-1447 (1989)).

15 The bacterial and animal GS sequences are not  
homologous. The structural genes for the bacterial  
GS are mapped to pOP12 in E.coli and glycogen  
synthase maps to glgA. Nucleotide sequencing  
further refined the position of glgA. The  
20 translation start point of glgA is known to be  
immediately following glgC and the nucleotide  
sequence determined. The NH<sub>2</sub> sequence was known so  
that the actual start of the glgA gene was  
unambiguously determined as well as confirming the  
25 direction of transcription. The deduced amino acid  
sequence shows complete homology with the known NH<sub>2</sub>  
sequence and with the known amino acid sequence.  
Different bacterial enzymes show 90% homology.  
There is complete agreement between the reported  
30 and deduced amino acid sequences for the enzyme.  
Cells transformed with the gene produce a  
polypeptide that has sequence homology with the  
known amino acid sequences.

Figure 18 shows the nucleotide sequence for



E.coli glycogen synthase as retrieved from EMBL #J02616. It is not a large protein: the structural gene is 1431 base pairs in length, specifying a protein of 477 amino acids with an estimated molecular weight of 49,000. It is known that problems of codon usage can occur with bacterial genes inserted into plant genomes but this is generally not so great with E.coli genes as with those from other bacteria such as those from Bacillus. Glycogen synthase from E.coli has a codon usage profile much in common with maize genes but it is preferred to alter, by known procedures, the sequence at the translation start point to be more compatible with a plant consensus sequence:

15        glgA --- G A T A A T G C A G  
         cons --- A A C A A T G G C T

The GS gene construct requires the presence of an amyloplast transit peptide to ensure its correct localisation in the amyloplast. It is believed that chloroplast transit peptides have similar sequences but other potential sources are available such as that attached to ADPG pyrophosphorylase (Plant Mol. Biol. Reporter (1991) 9, 104-126). Other potential transit peptides are those of small subunit RUBISCO, acetolactate synthase, glyceraldehyde-3P- dehydrogenase and nitrite reductase. For example,

25        Consensus sequence of the transit peptide of small subunit RUBISCO from many genotypes has the sequence:

30        MASSMLSSAAV--ATRTNPAQAS MVAPFTGLKSAAFPVSRK  
         QNLDITSIA SNGGRVQC

and the corn small subunit RUBISCO has the sequence:

MAPTVMMASSAT-ATRTNPAQAS AVAPFQGLKSTASLPVARR  
SSRSLGNVA SNGGRIRC

5 The transit peptide of leaf starch synthase from corn has the sequence:

MA ALATSQLVAT RAGLGVPDAS TFRRGAAQGL RGARASAAAD  
TLSMRTASARA APRHQQQARR GGRFPSLVVC

10 The transit peptide of leaf glyceraldehyde-3P-dehydrogenase from corn has the sequence:

MAQILAPS TQWQMRITKT SPCATPITSK MWSSLVMKQT  
KKVAHSAKFR VMAVNSENGT

The putative transit peptide from ADPG pyrophosphorylase from wheat has the sequence:

15 RASPPSESRA PLRAPQRSAT RQHQRQGPR RMC

It is possible however to express the glycogen synthase constitutively using one of the well-known constitutive promoters such as CaMV35S but there may be biochemical penalties in the plant resulting from increased starch deposition throughout the entire plant. Deposition in the endosperm is much preferred.

Possible promoters for use in the invention include the promoters of the starch synthase gene, ADPG pyrophosphorylase gene, and the sucrose synthase gene.

25 Figure 19 herewith illustrates the construction of two glycogen synthase vectors for use in this invention to transform tomato. Vectors are described with either constitutive or fruit-specific promoters. The transit peptide from tomato ribulose-bis-phosphate carboxylase is incorporated to direct the product to the plastid.

USE OF BACTERIAL BRANCHING ENZYME

Another embodiment of this invention is to use a temperature stable form of branching enzyme which can be obtained from bacteria. Branching enzyme  
5 [1,4- $\alpha$ -D-glucan: 1,4- $\alpha$ -D-glucan  
6- $\alpha$ -D-(1,4- $\alpha$ -D-glucano) transferase (E.C.  
2.4.1.18)] converts amylose to amylopectin, (a  
segment of a 1,4- $\alpha$ -D-glucan chain is transferred to  
a primary hydroxyl group in a similar glucan chain)  
10 sometimes called Q-enzyme. Like soluble starch  
synthase, this reaction also has  
temperature-dependent properties in plants,  
presumably because of the same molecular mechanisms  
of helix-to-chain transitions. It is reasonable to  
15 believe that the bacterial BE enzyme will behave  
similarly.

The most favoured sources of the branching  
enzyme gene for use in this invention are bacterial  
although plant enzymes can also be used (rice  
20 endosperm, Nakamura et al., *Physiologia Plantarum*  
84, 329-335 (1992); pea embryo, Smith, *Planta* 175,  
270-279 (1988); maize endosperm, Singh and Preiss,  
*Plant Physiology* 79, 34-40 (1985); Vos-  
Scherperkeuter et al., *Plant Physiology* 90, 75-84  
25 (1989)). The nucleotide and amino acid sequences  
for bacteria are known from the literature (Kiel  
JAKW et al, 1991, *Mol Gen Genet*, 230(1-2):136-144).

The structural genes for the bacterial BE are  
mapped to pOP12 in *E.coli* and branching enzyme maps  
30 to glgB.

The BE gene construct may require the presence  
of an amyloplast transit peptide to ensure its  
correct localisation in the amyloplast, as  
discussed previously for the glycogen synthase gene

construct.

It is possible to express the branching enzyme constitutively using one of the well-known constitutive promoters such as CaMV35S but there  
5 may be biochemical penalties in the plant resulting from increased starch deposition throughout the entire plant. Deposition in the endosperm is much preferred.

Possible promoters for use in the invention  
10 include the promoters of the starch synthase gene, ADPG pyrophosphorylase gene, and the sucrose synthase gene.

Branching enzyme vectors may be used to transform corn, with either constitutive or  
15 endosperm-specific promoters. The transit peptides from corn amyloplast-specific enzymes may be incorporated to direct the product to the plastid.

#### 20 EFFECTS ON STARCH FINE STRUCTURE

Although this invention is directed primarily to improvement of the deposition of starch at elevated climatic temperatures, alteration of starch deposition inevitably leads to alteration of  
25 starch fine structure.

In cereal crops, changing the ratios and activities of SSS and BE and/or the source of the enzymes (eg replacing maize SSS with pea SSS) alters the fine-branching structure of the starch.  
30 For example, the fine branching structure of starch is determined by the overall activities of the various isoforms of the SSS and BE enzymes being expressed during starch deposition in the developing endosperm. Altering the ratios of these

isoforms may be achieved by transformation techniques in which some of the natural enzyme activities are repressed whilst others are over-expressed in a manner analogous to the changes reported herein for the starch mutants of corn.

#### EFFECTS ON TEXTURE

Improved starch deposition also leads to alteration of the texture of crops such as tomatoes because increased amounts of starch in the fruit would increase the total solids content.

This effect would be significant in tomatoes which are grown for processing into paste. The quality of paste produced from processed tomatoes is in part related to the viscosity of the product which is usually determined by the Bostwick flow rate, reduced flow rate being desirable. The factors that interact to give a thicker product with reduced flow rate are complex, involving interactions between insoluble and soluble components. It is important to note that the characteristics of components in whole fruit will change during processing because of enzyme action and chemical changes brought about by heating which is involved in tomato processing by the so-called "hot-break" method.

The consistency of hot break paste is likely to be improved by increasing the level of insoluble solids in the whole fruit used in processing. Increased levels of soluble and insoluble solids in processing tomatoes has been an object of plant breeders for many years.

Soluble solids are the solutes in the tomato

serum and consists primarily of carbohydrates. Paste is normally sold on the basis of its natural tomato soluble solids (NTSS) content. Because the sugars are the major contributors to NTSS, a higher sugar content contributes to a higher yield of paste per tonne of tomatoes. The correlation between NTSS and total solids (TS) is very high, although the relationship varies amongst tomato cultivars. High NTSS levels in ripe fruit may be an indirect measure of the starch component of the insoluble solids during fruit development. Sugar content is a critical component of the flavour of tomatoes.

Insoluble solids (IS) consist mainly of the polysaccharides in the cell wall. Residual starch will also contribute to the IS although, in normal ripening, this forms a small component. The IS/TS ratio partially determines the consistency of tomato products. Where high consistency is required, a greater quantity of IS improves the product quality. IS are measured as both water-insoluble solids (WIS) and alcohol-insoluble solids (AIS). The AIS quantities are greater than those for WIS because smaller polysaccharides are less soluble in 80% ethanol than in water.

It is believed that, by the method of this invention, the increased amounts of starch may very well accumulate in plastid granules and are, therefore, unlikely to contribute much to improved consistency. However, the heating involved in the hot break process and in the concentration step of hot and cold break products is likely to burst the granules and partially solubilise the starch, resulting in increased viscosity.

Enhanced starch synthesis is also likely to elevate the import of carbohydrate into the fruit which may also result in enhanced levels of soluble solids in the ripe fruit. This may also be  
5 considered as advantageous in fruit intended for the fresh fruit market.

In summary, then, improved insoluble solids leads to improved consistency of paste and higher yields of paste per tonne of tomato processed,  
10 whereas increased soluble solids will result in higher yields of paste per tonne of tomato processed and improved sugar component of flavour.

In tomato, the free sugars are almost entirely fructose and glucose. Sucrose is present but  
15 rarely exceeds 1% of the dry weight. Starch and structural polysaccharides are the major forms of storage of imported carbon. Starch levels increase in the early stages of fruit development, followed by a decrease to virtually zero by ripeness. Thus,  
20 in ripe fruit, hexoses (glucose and fructose) are the primary component of the soluble solids and account for about 50% of the fruit dry weight.

Thus, from the known facts about the ripening process it is believed that increasing the soluble  
25 solids is advantageous and that this increase may be obtained by transformation with a gene encoding glycogen synthase.

Our invention is thus applicable to plants whose value is due to the texture and/or sweetness  
30 and/or taste of the fruit. For example fruits such as tomato, melon, peach, pear, etc where the accumulation of starch is an early event in fruit development but is later degraded during fruit ripening releasing sugars. In this case the

desirable trait is extra starch deposition which is useful in providing enhanced fruit texture and sweetness as well as increased thickening quality during cooking (eg, during ketchup/catsup manufacture from tomato).

#### OTHER USEFUL EFFECTS

Starch deposition in the developing pollen grains of cereals is an essential prerequisite for pollen viability. If starch synthesis is impaired by high temperatures in the developing pollen cells, reduced pollen viability will result. The insertion, according to the invention, of thermally stable variants of the SSS and/or BE and/or GS enzyme genes into cereal pollen increases pollen viability and seed set is less impaired by exposure to high temperatures.

Starch synthesis in leaf chloroplasts is also limited by the thermal lability of the SSS and BE enzymes. Photosynthetic rates in green leaf tissue are dependent in part on the ability of the cells to convert fixed carbon into starch and sucrose. When starch synthesis becomes limited at high temperatures because of rate limitation by SSS or BE, there is an accumulation of metabolic intermediates in the chloroplast, causing feedback inhibition of ribulose biphosphate regeneration, reducing the overall carbon fixation by photosynthesis. Insertion of thermally stable variants of the enzymes, or additional copies of the relevant gene(s), increases the photosynthetic rate at high temperatures. This will increase the yield of all crops whose yield is dependent on the



photosynthetic rate of the source leaves during at least a part of their life cycle. Examples include cereal crops such as maize, sorghum, wheat, barley and rice; root crops such as potato, turnip, yam and cassava; sugar crops such as beet and cane; oilseed crops such as rapeseed, sunflower, oil palm, coconut, linseed and groundnut; fruits such as apples, pears and bananas; and meal crops such as soya, beans and peas. The yields of all of these crops may be limited by source activity at particular times in their life cycle.

Our invention is also applicable to plants whose yield depends on gross biomass accumulation and which are limited by photosynthetic rate. Examples are forage crops such as grasses, ryegrass, forage maize and alfalfa; trees grown for wood, pulp or ethanol production and vegetables such as cauliflower, cabbage and sprouts.

#### CROSS-BREEDING

In one specific aspect, our invention is a method of producing a novel substantially homozygous maize (corn) line having superior starch deposition properties which comprises, (i) identifying a range of potential donor plants which are sexually compatible with a recipient maize plant and screening producing soluble starch synthase (SSS) enzyme or branching enzyme (BE) to determine the heat stability of the reaction of at least one of said enzymes; (ii) identifying a plant producing an enhanced SSS or BE enzyme that is significantly more heat-stable for the enzyme reaction than the corresponding enzyme in the

recipient maize plant; (iii) crossing the identified plant with the recipient maize plant; (iv) selecting from among the progeny those expressing the heat stable enzyme reaction of the donor; and, (v) breeding therefrom so as to produce a novel substantially homozygous maize line having an enhanced rate of starch deposition.

Genotypes with a measurable enhancement in SSS or BE activity or altered characteristics with respect to temperature, are introduced into a back-crossing programme with a commercial maize inbred. Progeny are selected on the basis of genetic similarity to the commercial line, using RFLP's (restriction fragment length polymorphisms), but with the desired SSS or BE characteristics. Selected progeny are entered into further back-crossing against the commercial line. The end result is a new maize inbred line, genetically very similar to the parental line, having enhanced SSS or BE activity. The temperature optimum of starch synthesis is measured before including the new line in hybrid production.

Another embodiment of our invention is a method of producing a novel substantially homozygous maize (corn) line having superior starch deposition properties which comprises, (i) identifying a range of potential donor plants which are sexually compatible with a recipient maize plant and screening for plants with high rates of grain starch synthesis at elevated temperatures; (ii) identifying a plant producing an enhanced rate of starch deposition that is significantly more heat-stable than the corresponding rate in the recipient maize plant; (iii) crossing the

- identified plant with the recipient maize plant;  
(iv) selecting from among the progeny those  
expressing the high rate of starch deposition; and,  
(v) breeding therefrom so as to produce a novel  
5. substantially homozygous maize line having an  
enhanced rate of starch deposition.

Genotypes with a measurable enhancement in  
starch deposition rates with respect to temperature  
are introduced into a back-crossing programme with  
10 a commercial maize inbred. Progeny are selected on  
the basis of genetic similarity to the commercial  
line, using RFLP's (restriction fragment length  
polymorphisms), but with the desired SSS or BE  
characteristics. Selected progeny are entered into  
15 further back-crossing against the commercial line.  
The end result is a new maize inbred line,  
genetically vary similar to the parental line,  
having enhanced SSS or BE activity. The  
temperature optimum of starch synthesis is measured  
20 before including the new line in hybrid production.

#### EXAMPLE 1

##### Sexual Crossing

The plants selected for screening were maize  
25 plants: either commercial maize varieties or  
varieties from more exotic collections. The  
material selected for screening was from amongst  
other Zea germplasm, for example, Zea tripsacum,  
perennis, diploperennis, luxurians, parviglumis,  
30 mexicana and mays. Many thousands of potential  
donors exist throughout the world where maize is  
grown as a cultivated crop or where it exists in  
the wild plant population, for example in South and  
Central America and Africa. Most of the Zea

family can be inter-bred by traditional plant breeding methods.

One characteristic sought for use in this invention was an increase (or minimal loss) in activity from 25 to 35°C. A  $Q_{10}$  value for each line was obtained by a method which is hereinafter described. The frequency distribution for all the germplasm assayed in the screen (Figure 20) shows that the range of variation for this trait is quite narrow. However, our screen succeeded in locating a few rare occurrences from the extremely wide selection of Zea germplasm. Across all the germplasm the overall average drop in activity between 25 and 35°C was around 40% ( $Q_{10} = 0.61$ ) with a range of 65% to 0% drop in activity ( $Q_{10}$  range of 0.35 to 1.00). A selection of sixteen inbreds used in commercial hybrids showed a narrower range of 41% to 20% drop in activity ( $Q_{10}$  of from 0.59 to 0.80 with an average of 0.70) indicating that conventional maize breeding which involves selection of yield-for-moisture may possibly have been effective in weeding-out the very worst forms of the enzyme. However, there is clearly room for further improvement in several inbreds and hence it is possible to gain potential yield benefit in commercial hybrids.

The very best forms of SSS identified (from a screen of nearly 1,000 sources of Zea germplasm) where in 4 exotic lines from Peru (Lima 38 and Lima 45) and Mexico (Guanajuato 13) and teosinte with  $Q_{10}$  as high as 1.0. Three of these lines were obtained from the Plant Introduction Centre, Iowa State University: Numbers P1515021, Ames 8545, PI490879 and one teosinte line obtained from Dr

John Doebley Zea mays subsp. Mexicana, Doebley 479. The temperature/activity profiles (Figure 21) of these forms of the enzyme(s) show a dramatic difference from one commercially valuable inbred line (UE95) which was included for comparison.

These two exotic lines have been crossed into commercial inbred germplasm to produce F<sub>1</sub> hybrids.

#### GENE MANIPULATION

A donor gene may also be introduced into a recipient plant by transformation.

When genetic manipulation techniques are employed in this invention there are at least four possibilities:

(1) Increase the amount and activities of the enzyme SSS or BE or GS or any combination thereof in a recipient plant, such as a commercial maize line or population by the insertion of extra gene copies of the SSS, BE or GS enzymes. The source of these extra copies may be the recipient line itself as the technique would simply increase the amount of enzyme available in the grain rather than the changing of the properties of the enzyme(s). The gene promoters and other regulatory sequences may also be altered to achieve increased amounts of the enzyme in the recipient plant.

(2) The insertion of a gene or genes specifying SSS and/or BE and/or GS enzymes with activities which increase with temperature up to 30°C. Achieving this requires the identification of a source of the thermally stable enzyme gene. However, the use of genetic manipulation techniques

for introduction of the new genes places no restriction of the source to sexually compatible species and genetic material may be obtained from any source of the SSS, BE and GS enzymes having the  
5 desired temperature/activity characteristics.

Enzymes from plant species other than cereals, particularly plants which grow well in tropical or sub-tropical regions will most likely exhibit greater temperature stability in the SSS and BE  
10 enzymes than maize. The exogenous genetic material may altered by genetic engineering to give the desired characteristics to the enzymes. Techniques are known in so-called "protein engineering" which can alter the characteristics of an enzyme.

15 (3) Change the ratios of activities of the isoforms of enzymes SSS or BE or any combination thereof in a recipient plant, such as a commercial maize line or population by the insertion of extra gene copies of the SSS, BE enzymes and/or by  
20 insertion of anti-sense gene constructs. The source of these extra copies or antisense constructs may be the recipient line itself as the technique would simply increase or decrease the amount of enzyme available in the grain rather than  
25 the changing of the properties of the enzyme(s). The gene promoters and other regulatory sequences may also be altered to achieve increased amounts of the enzyme in the recipient plant.

(4) The insertion of a gene or genes  
30 specifying SSS and/or BE and/or GS enzymes with activities which effect a change in the fine structure of the starch. Achieving this requires the identification of a source of the enzyme gene. However, the use of genetic manipulation techniques

for introduction of the new genes places no restriction of the source to sexually compatible species and genetic material may be obtained from any source of the SSS, BE and GS enzymes having the desired characteristics. Enzymes from plant species other than cereals, particularly plants which grow well in tropical or sub-tropical regions will most likely exhibit altered starch specificities in the SSS and BE enzymes than maize. The exogenous genetic material may altered by genetic engineering to give the desired characteristics to the enzymes. Techniques are known in so-called "protein engineering" which can alter the characteristics of an enzyme.

#### ISOLATION OF A DONOR GENE

The donor gene may be isolated from a suitable biological organism (including plants, fungi, bacteria or animal cells) after screening a range of potential donor organisms producing soluble starch synthase (SSS) enzyme, glycogen synthase (GS) or branching enzyme (BE) to determine the heat stability of the reaction of at least one of said enzymes. (In bacteria or animals, glycogen is deposited rather than starch and so these organisms contain glycogen synthases and branching enzymes).

#### EXAMPLE 2

##### Screening for source material

We have devised a means (an enzyme-activity based screen) of identifying heat-stable forms of the enzymes. When the natural Zea mays temperature sensitive enzymes are replaced with temperature

stable enzymes from another organism, provided the replacement enzymes are not disrupted by low temperatures in the environment, maize plants with higher rates of starch synthesis at temperatures above 25°C will result.

The screen used in this invention identified enzyme forms with increased (or minimal loss in ) activity from 25 to 35°C. However, the characteristics sought for use in the invention can be any one, or several in combination, of the following characteristics:

- (1) a high increase in activity between 20 and 25°C;
- (2) increased (or minimal loss in ) activity from 25 to 30°C;
- (3) increased (or minimal loss in ) activity from 30 to 35°C;
- (4) increased stability to "knockdown", where knockdown is defined as an irreversible loss in activity caused by elevated temperatures; and,
- (5) intrinsically high activity at 20°C.

### EXAMPLE 3

#### Enzyme Activity Assay

In order to assess the suitability of the native enzymes of potential gene donor plants, it is necessary to be able to assay for the thermally stable enzymes. We have devised the following procedure.

1. Homogenise grain or endosperm tissue and extract protein into a buffer solution which maintains the SSS and BE enzymes in an active form.
2. Assay SSS activity by adding 50µl of the enzyme extract to 25µl of primer (glycogen,



amylopectin, starch) and 100 $\mu$ l of a buffer solution of the following final concentrations: 100mM bicine, 25mM potassium chloride, 5mM EDTA, and 10mM reduced glutathione.

- 5     3.    Begin the assay by adding  $^{14}\text{C}$ -ADP-glucose. Stop the assay after a defined time at a defined temperature by adding 1ml of 0.1M sodium hydroxide. Precipitate the primer by bringing the mixture to 75% methanol concentration. Centrifuge and recover  
10    the primer pellet and redissolve in sodium hydroxide and reprecipitate in 75% methanol. Repeat the reprecipitation procedure.
- 15    4.    Following the second wash, dissolve the primer in 1M hydrochloric acid at 100°C. Cool and add the mixture to scintillation fluid and measure the  
       $^{14}\text{C}$ -ADP-glucose transferred to the primer.
5.    BE activity is assayed by adding 50 $\mu$ l of the enzyme extract to a mixture containing citrate buffer, AMP, and phosphorylase A.
- 20    6.    Start the reaction by adding an aliquot of  $^{14}\text{C}$ -glucose-1-phosphate. Stop the reaction after a defined time at a selected temperature by adding 0.1M sodium hydroxide.
- 25    7.    Add a polysaccharide (amylose, glycogen, amylopectin, starch) as a carrier and precipitate the polysaccharide with 75% methanol. Centrifuge and wash the polysaccharide precipitate and  
      dissolve and count the scintillation as described in step 4 above. BE activity is defined as the  
30    stimulation achieved by the enzyme in the incorporation of  $^{14}\text{C}$ -glucose from the glucose-1-phosphate into the polysaccharide by the phosphorylase A.

## EXAMPLE 4

Isolation of soluble starch synthase (SSS) and branching enzyme (BE) genes

5 Using standard cloning techniques, the SSS and BE genes may be isolated.

10 The source of the genes was a US sweet-corn line of Zea mays, from which the enzyme protein was purified. Endosperms from the maize line were homogenised in a buffer which maintains the SSS and BE in active form. The enzymes were partially purified by ammonium sulphate fractionation, followed by DEAE Sepharose chromatography, followed by FPLC using a Superose gel filtration column, followed by FPLC using a Mono Q anion exchange column. The Superose column allows separation of SSS from BE activity. Further purification of individual isoforms was achieved by hydroxyapatite, cation exchange FPLC or isochromatofocussing.

20 Purification of the SSS from maize (Silver Queen) has been achieved (Wasserman et al, Plant Physiology, (in press)) by a combination of ammonium sulphate precipitation, ion exchange chromatography, affinity chromatography (Affi-Gel Blue) and two FPLC steps, Mono-Q and Superose-12. This results in up to 5,000-fold purification with yields up to 5%. The SSS polypeptide was a single subunit of molecular weight 86kD. Other SSS polypeptides were present in a US dent inbred line at around 70kD and 105kD molecular weight.

30 Ammonium sulphate precipitation of SSS I is best achieved using 40% ammonium sulphate which produces a translucent SSS-enriched pellet which is next dialysed and further fractionated using DEAE-cellulose ion-exchange chromatography. These

steps increase specific activities by up to 50-fold. The affinity chromatography steps rely on the ADP attached to a Sepharose matrix either through the N-amino group or through The ribose hydroxyl group. SSS I is readily eluted with 2mM ADP at pH 8.5. Specific activities are increased by up to 10-fold using the affinity chromatography step with a yield of around 15%. SSS is next purified by Mono-Q FPLC steps with elution of activity at low potassium chloride (not more than 200mM KCl).

Purification of the SSS and BE enzymes from the US inbred line identified three SSS and three BE isoforms. Figure 22 shows the data for SSS; BE behaves similarly. Preliminary investigations have suggested that these isoforms have slightly different temperature optima of activity and also slightly different temperature thresholds for knockdown.

In the final purification step the SSS or BE preparations were loaded on to SDS PAGE gels. The bands corresponding to the SSS or BE polypeptides were cut out and eluted. The pure polypeptide was then used as an antigen to generate polyclonal antibodies in a rabbit. The antibodies were then tested for specificity to the SSS or BE polypeptides. N-terminal amino acid sequences were also obtained from the polypeptides.

Full sequencing of the maize polypeptides is continuing. Amino acid sequencing of the maize SSS polypeptide has provisionally yielded the following partial sequence: Ala-Ala-xxx-Arg-Lys-Ala-Val-Met-Val-Pro-xxx-Gly-xxx-Asn-Arg-Glu-Phe-Val-Lys-Tyr-Leu-Phe-xxx-Met/Phe-Ala-Gln. The final sequence may

be compared to the amino acid sequence of pea SSS I and SSS II published by Dry et al (1991, Plant Journal, 2:193-202).

5 The antibodies may be used to screen a maize endosperm cDNA library for clones derived from the mRNAs for SSS or BE in an in vitro transcription/translation system.

10 The cDNAs thus derived may be used to probe a maize genomic library and the maize SSS and BE genomic DNAs may be isolated. In addition N-terminal amino acid sequence information for SSS and BE may be used to generate oligonucleotide probes. These probes may be used to screen the maize genomic library and the maize SSS and BE  
15 genomic DNAs may be isolated.

#### TRANSFORMATION

##### (i) Insertion of extra copies of the gene

20 Maize genomic DNAs isolated as above may subsequently be transformed into either protoplasts or other tissues of a maize inbred line or population. The existing gene promoters ensure that the extra genes are expressed only in the  
25 developing endosperm at the correct developmental time. The protein sequences likewise ensure that the enzymes are inserted into the amyloplast.

Transgenic maize plants are regenerated and the endosperms of these plants are tested for  
30 increased SSS and BE enzyme activity. The kernels are also tested for enhanced rate of starch synthesis at different temperatures. The plants are then included in a breeding programme to produce new maize hybrids with higher rates of

starch synthesis at temperatures above the normal optimum.

- 5           (ii) Insertion of genes specifying SSS and/or  
BE with higher temperature optima for activity.

          This is also achieved by standard cloning techniques. The source of the temperature-stable forms of the SSS or BE or GS genes is any organism that can make starch or glycogen. Potential donor  
10       organisms are screened and identified as described above. Thereafter there are two approaches:

- (a) via enzyme purification and antibody/sequence generation using the protocol described above.  
          (b) using SSS and BE and GS cDNAs as heterologous  
15       probes to identify the genomic DNAs for SSS and BE and GS in libraries from the organism concerned. The gene transformation, plant regeneration and testing protocols are as described above. In this instance it is necessary to make gene constructs  
20       for transformation which contain the regulatory sequences from maize endosperm SSS or BE or another maize endosperm starch synthesis pathway enzyme to ensure expression in endosperm at the correct developmental time (eg, ADPG pyrophosphorylase).

- 25           One specific example of this is with the bacterial glycogen synthase enzyme which we have found to be essentially tolerant of temperatures up to 40°C. The nucleotide and amino-acid sequences of glycogen synthase are known (i) from E Coli  
30       GenBank/EMBL #JO2616 (Kumar et al, J Biol Chem 34 16256-16259 (1986)). (ii) from rabbit skeletal muscle (Zhang et al, FASEB J 3 2532-2536 1989)), and (iii) from human muscle (Browner et al, Proc Nat Acad Sci 86 1443-1447 (1989)). Gene constructs

used to transform plants requires the regulatory sequences from maize endosperm SSS or BE or another maize endosperm starch synthesis pathway enzyme to ensure expression in endosperm at the correct development time (eg, ADPG pyrophosphorylase).  
5 Furthermore the gene constructs also requires a suitable amyloplast transit-peptide sequence such as from maize endosperm SSS or BE or another maize endosperm starch synthesis pathway enzyme to  
10 ensure expression of the amyloplast at the correct developmental time (eg, ADPG pyrophosphorylase).

Genetic protein engineering techniques may also be used to alter the amino acid sequence of the SSS or BE or GS enzymes to impart higher  
15 temperature optima for activity. The genes for SSS and/or BE and/or GS may be cloned into a bacteria which relies on these enzymes for survival. Selection for bacteria surviving at evaluated temperatures enables the isolation of mutated  
20 thermostable enzyme forms. Transformation of maize with the altered genes is carried out as described above.

Genetic protein engineering techniques may also be used to alter the amino acid sequence of the maize SSS or BE enzymes to impart higher  
25 temperature optima for activity. The genes for SSS and/or BE may be cloned into bacteria relies on the these enzymes for survival. Selection for bacteria surviving at elevated temperatures enables the  
30 isolation of mutated thermostable enzymes forms. Transformation of maize with the altered genes is carried out as described above.

(iii) Changing the ratios of activities of the isoforms of enzymes SSS or BE.

This is also achieved by standard cloning techniques. The source of the SSS or BE genes is  
5 maize using the protocol described above. Plants are then transformed by insertion of extra gene copies of the isoforms of SSS, BE enzymes and/or by insertion of anti-sense gene constructs. The gene promoters and other regulatory sequences may also  
10 be altered to achieve increased amounts of the enzyme in the recipient plant.

(iv) Insertion of a gene or genes specifying SSS and/or BE and/or GS enzymes with activities which effect a change in the fine structure of the starch.

This is also achieved by standard cloning techniques. The source of the special forms of the SSS or BE or GS genes is any organism that can make  
20 starch or glycogen. Potential donor organisms are screened and identified as described above.

Thereafter there are two approaches:

- (a) via enzyme purification and antibody/sequence generation using the protocol described above.
- 25 (b) using SSS and BE and GS cDNAs as heterologous probes to identify the genomic DNAs for SSS and BE and GS in libraries from the organism concerned. The gene transformation, plant regeneration and testing protocols are as described above. In this  
30 instance it is necessary to make gene constructs for transformation which contain the regulatory sequences from maize endosperm SSS or BE or another maize endosperm starch synthesis pathway enzyme to ensure expression in endosperm at the correct  
35 developmental time (eg, ADPG pyrophosphorylase).

CLAIMS

1. A method of producing a plant with altered starch synthesising ability comprising stably incorporating into the genome of a recipient plant one or more than one donor gene  
5 specifying an enzyme involved in a starch or glycogen biosynthetic pathway.
2. A method as claimed in claim 1 in which the plant has an improved capacity to produce  
10 starch at elevated or lowered temperature.
3. A method as claimed in claim 1 or claim 2 in which the plant has an ability to synthesise starch with an altered fine structure.  
15
4. A method as claimed in any of claims 1 to 3 in which the temperature optimum of starch synthesis is increased or decreased.
- 20 5. A method as claimed in any of claims 1 to 4 in which at least one of the donor genes is derived from a plant.
- 25 6. A method as claimed in claim 5 in which the donor gene is derived from a plant of the species Zea mays, Zea diploperennis, Zea luxurians, Zea perennis, Zea tripsacum, Zea parviglumis, Zea mexicana or teosinte.
- 30 7. A method as claimed in claim 6 in which the donor gene is derived from a plant of the Zea mays varieties Lima 38, Guanajuato 13, Lima 45, Doebley 479 or teosinte 154.



8. A method as claimed in any of claims 1 to 7 in which at least one of the donor genes is derived from a bacterium.
- 5 9. A method as claimed in any of claims 1 to 8 in which at least one of the donor genes is derived from a fungus.
- 10 10. A method as claimed in any of claims 1 to 9 in which at least one of the donor genes is derived from an animal cell.
- 15 11. A method as claimed in any of claims 1 to 10 in which the donor gene specifies soluble starch synthase (E.C. 2.4.1.21) and/or branching enzyme (E.C. 2.4.1.18) and/or glycogen synthase of bacterial origin (E.C. 2.4.1.11) or of animal origin (E.C. 2.4.1.21).
- 20 12. A method as claimed in any of claims 1 to 11 in which at least one of the donor genes specifies a modified allelic form of the enzyme.
- 25 13. A method as claimed in any of claims 1 to 12 in which at least one of the donor genes specifies a mRNA antisense to the mRNA encoded by the wild-type gene.
- 30 14. A method as claimed in any of claims 1 to 13 in which at least one of the donor genes is derived from a sexually compatible donor plant and is inserted into the recipient plant by sexual crossing of donor and recipient plants.

15. A method as claimed in any of claim 1 to 14 in which at least one of the donor genes is incorporated into the recipient genome by genetic transformation.
- 5
16. A method as claimed in any of claims 1 to 15 in which the recipient plant is of the family Gramineae.
- 10
17. A method as claimed in claim 16 in which the recipient plant is of the species Zea mays.
18. A method as claimed in any of claims 1 to 15 in which the recipient plant is a tomato.
- 15
19. A plant having one or more than one donor gene specifying an enzyme involved in a starch or glycogen biosynthetic pathway stably incorporated into its genome such that its ability to synthesise starch is altered.
- 20
20. A plant as claimed in claim 19 which has an improved capacity to produce starch at elevated or lowered temperature.
- 25
21. A plant as claimed in claim 19 or claim 20 which has an ability to synthesise starch with an altered fine structure.
- 30
22. A plant as claimed in any of claims 19 to 21 in which starch synthesis has an increased or decreased temperature optimum.

23. A plant as claimed in any of claims 19 to 22 in which at least one of the donor genes is derived from a plant.
- 5 24. A plant as claimed in claim 23 in which the donor gene is derived from a plant of the species Zea mays, Zea diploperennis, Zea luxurians, Zea perennis, Zea tripsacum, Zea parviglumis, Zea mexicana or teosinte.
- 10 25. A plant as claimed in claim 24 in which the donor gene is derived from a plant of the Zea mays varieties Lima 38, Guanajuato 13, Lima 45, Doebley 479 or teosinte 154.
- 15 26. A plant as claimed in any of claims 19 to 25 in which at least one of the donor genes is derived from a bacterium.
- 20 27. A plant as claimed in any of claims 19 to 26 in which at least one of the donor genes is derived from a fungus.
- 25 28. A plant as claimed in any of claims 19 to 27 in which at least one of the donor genes is derived from an animal cell.
- 30 29. A plant as claimed in any of claims 1 to 10 in which the donor gene specifies soluble starch synthase (E.C. 2.4.1.21) and/or branching enzyme (E.C. 2.4.1.18) and/or glycogen synthase (E.C. 2.4.1.11 or E.C. 2.4.1.21).

- 5
30. A plant as claimed in any of claims 19 to 29 in which at least one of the donor genes specifies a modified allelic form of the enzyme.
- 10
31. A plant as claimed in any of claims 19 to 30 in which at least one of the donor genes specifies a mRNA antisense to the mRNA encoded by the wild-type gene.
- 15
32. A plant as claimed in any of claims 19 to 31 which is of the family Gramineae.
- 20
33. A plant as claimed in claim 32 which is of the species Zea mays.
- 25
34. A plant as claimed in any of claims 19 to 31 which is a tomato.
35. A plant as claimed in any of claims 19 to 34 having a starch synthesising ability which does not decrease with temperature between 25 to 30°C.
36. A plant as claimed in any of claims 19 to 35 having a donor gene encoding soluble starch synthase and/or branching and/or glycogen synthase enzyme with a  $Q_{10}$  value greater than 0.8 between 25 and 35°C.

- 5 37. A plant as claimed in any of claims 19 to 36  
having a donor gene encoding soluble starch  
synthase and/or branching and/or glycogen  
synthase enzyme which is resistant to  
reduction of activity after exposure to a  
temperature in excess of 40°C for two hours.
- 10 38. Seeds of a plant as claimed in any of claims  
19 to 37.
39. A plant which is derived from a plant as  
claimed in any of claims 19 to 37.
- 15 40. A plant as claimed in claim 39 which is a  
hybrid.

1 / 27

Effect of changing 'rate' and 'duration' on final grain weight.

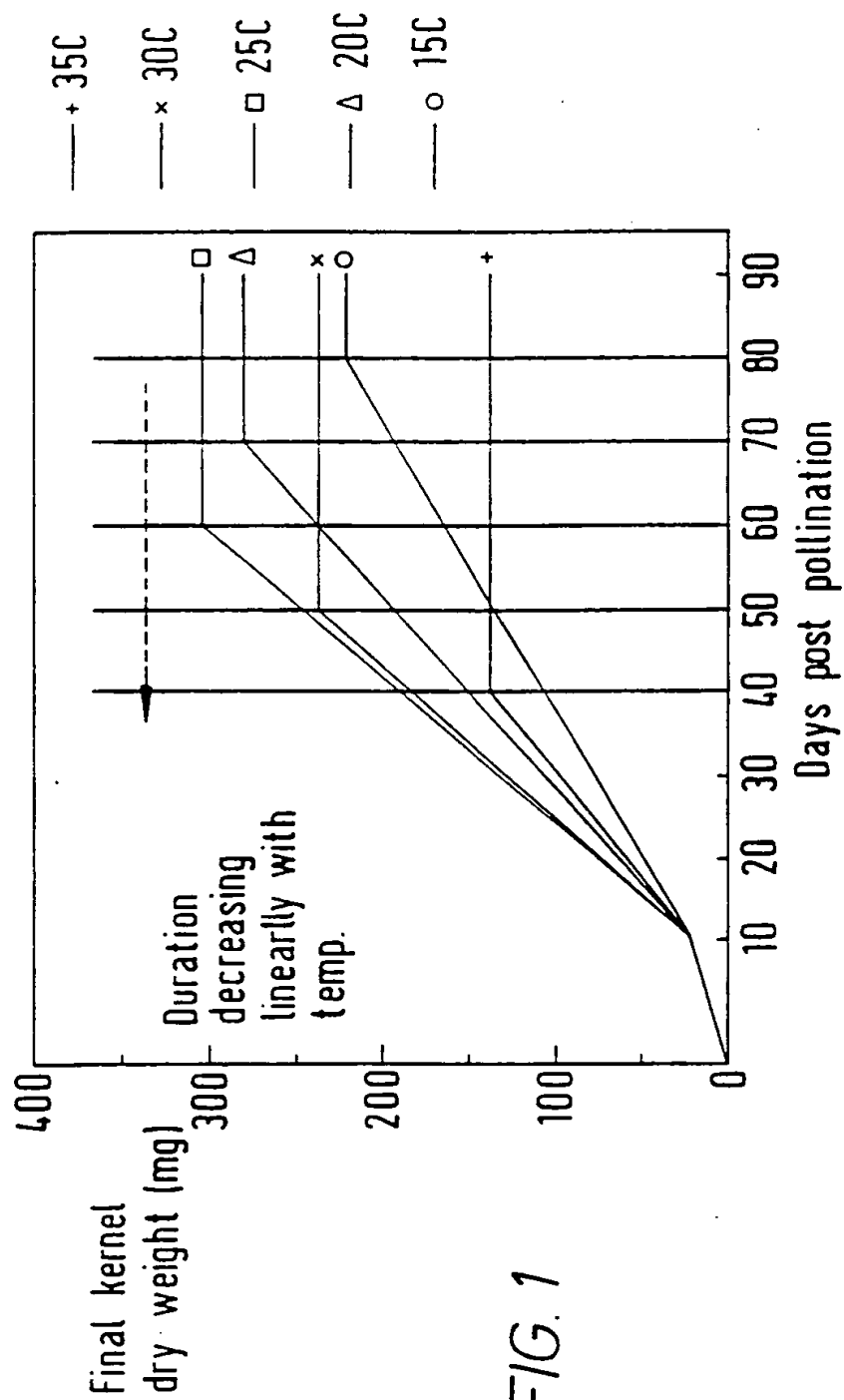
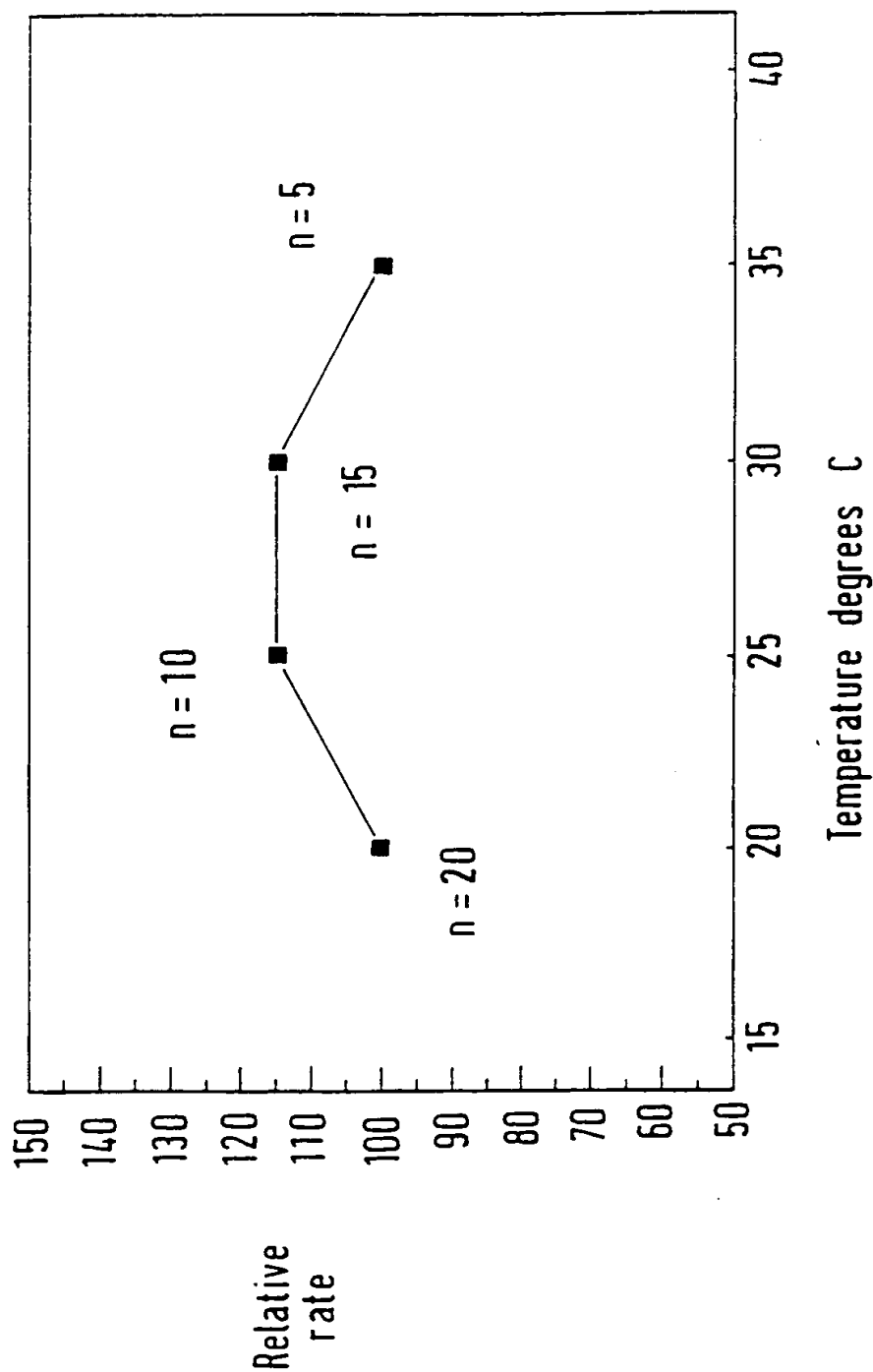


FIG. 1

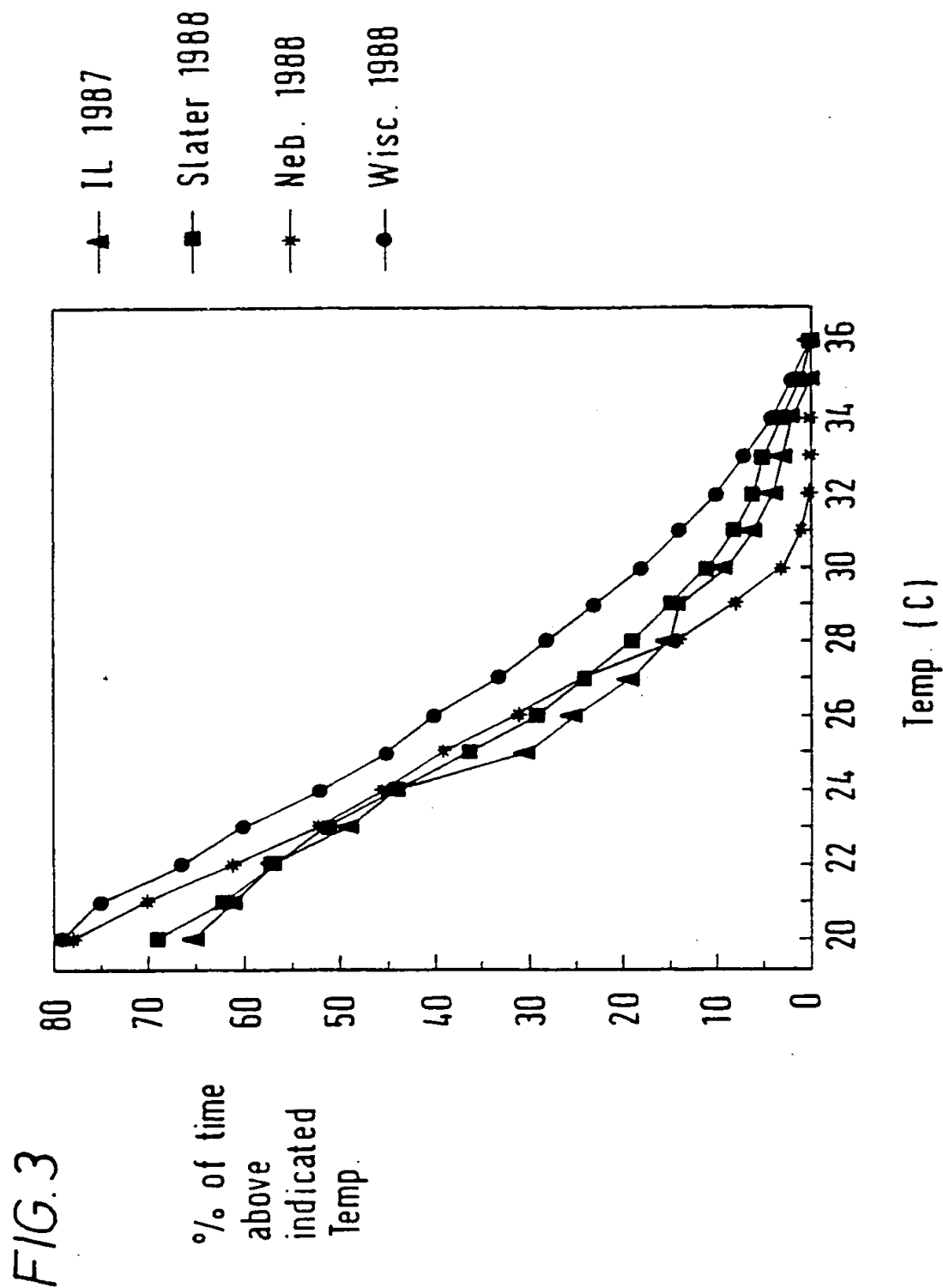
Starch Synthesis  
In-vivo rates

FIG. 2



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3/27

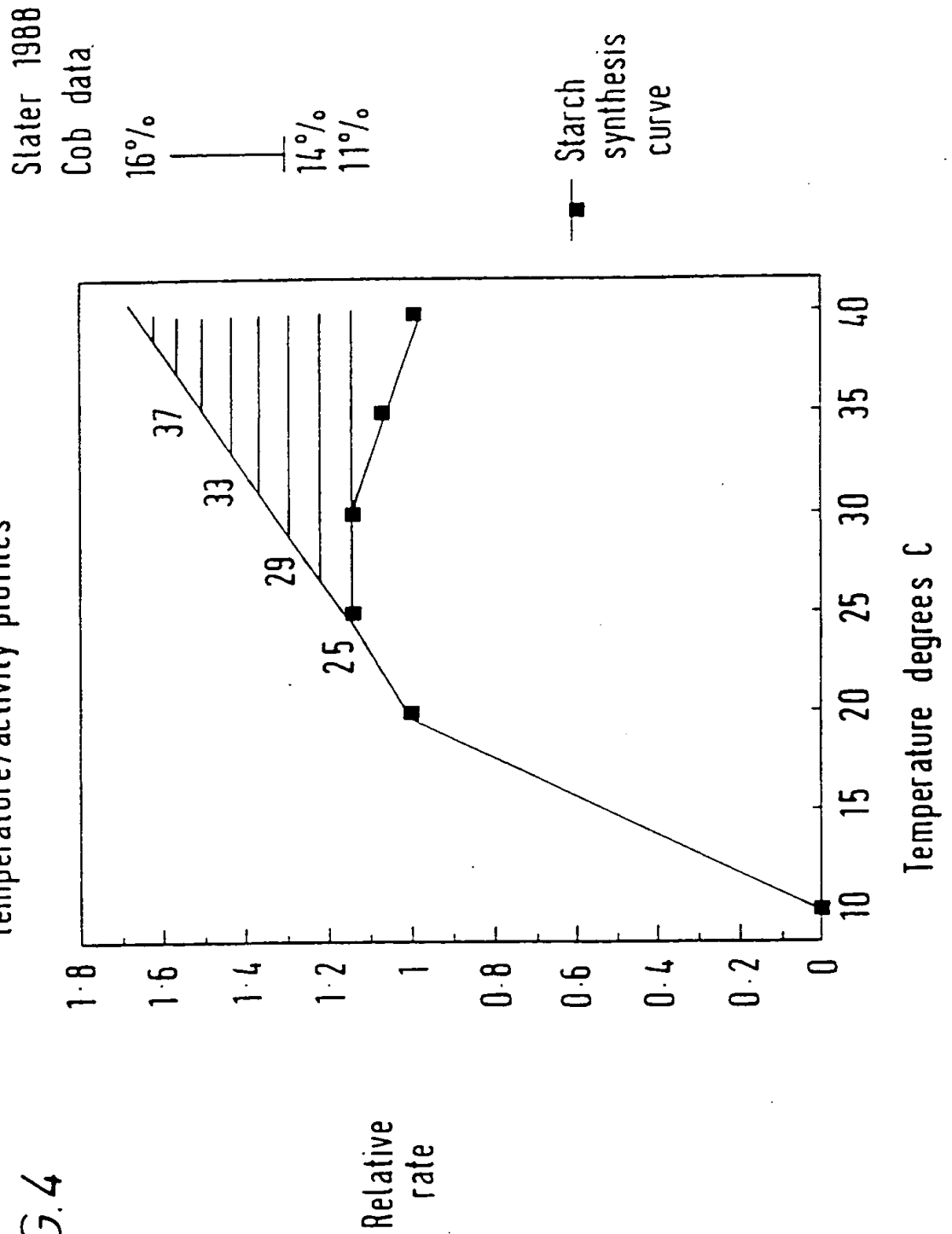


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Computer simulations of different rate - models of temperature/activity profiles

FIG. 4



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5/27

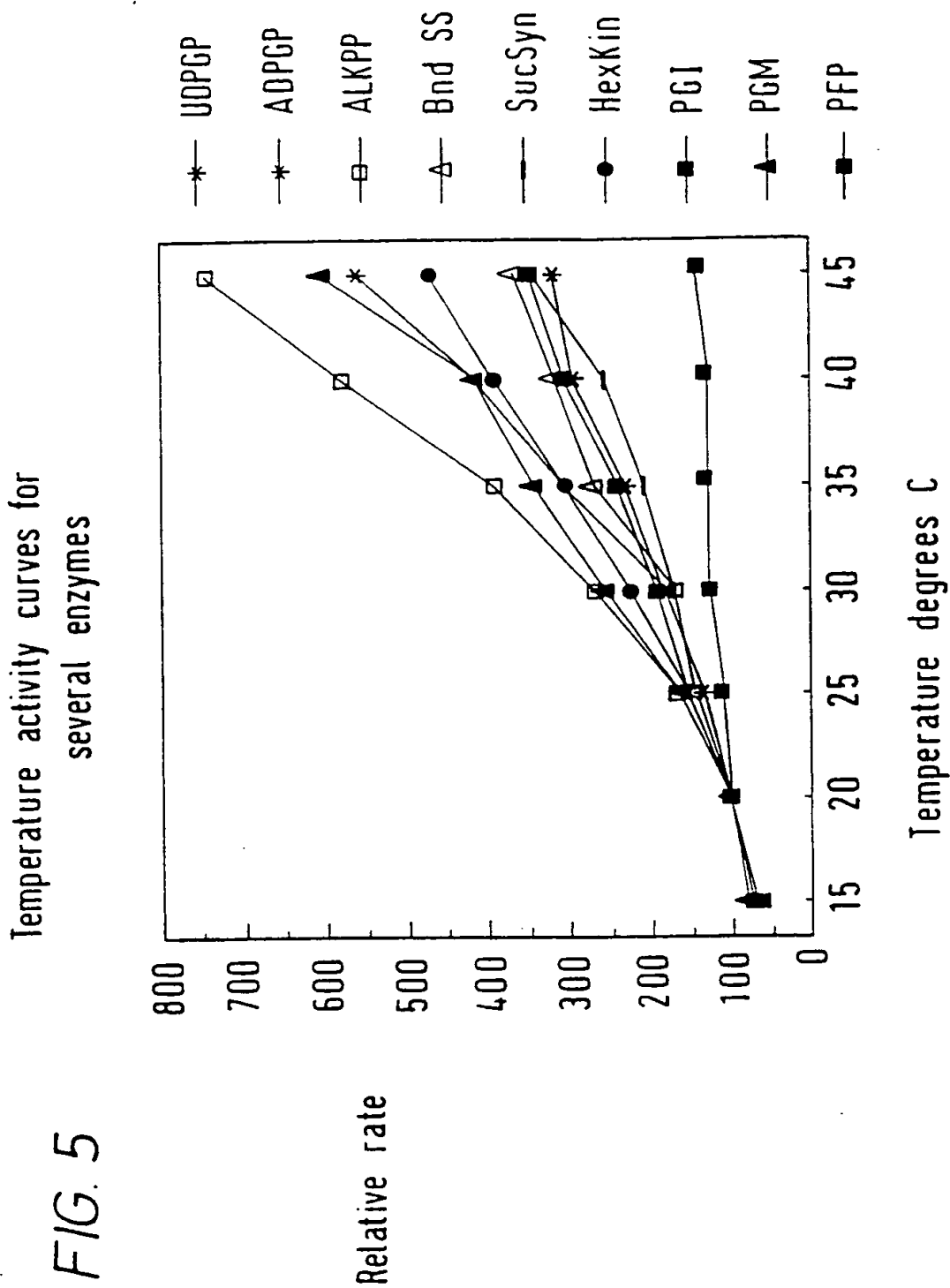
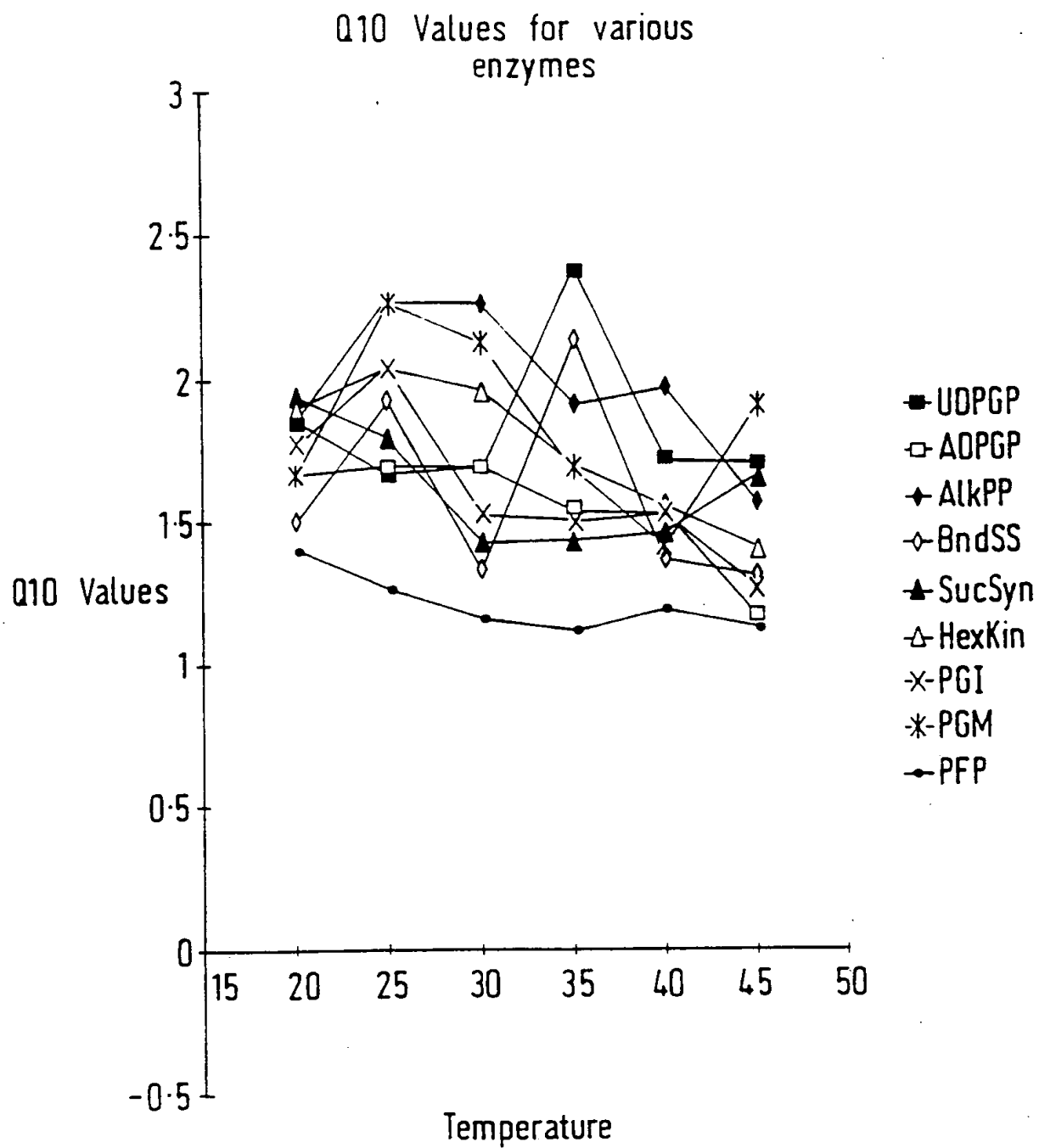


FIG. 5

6/27

FIG. 6



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7 / 27

Maize endosperm soluble starch synthase  
Maize endosperm branching enzyme

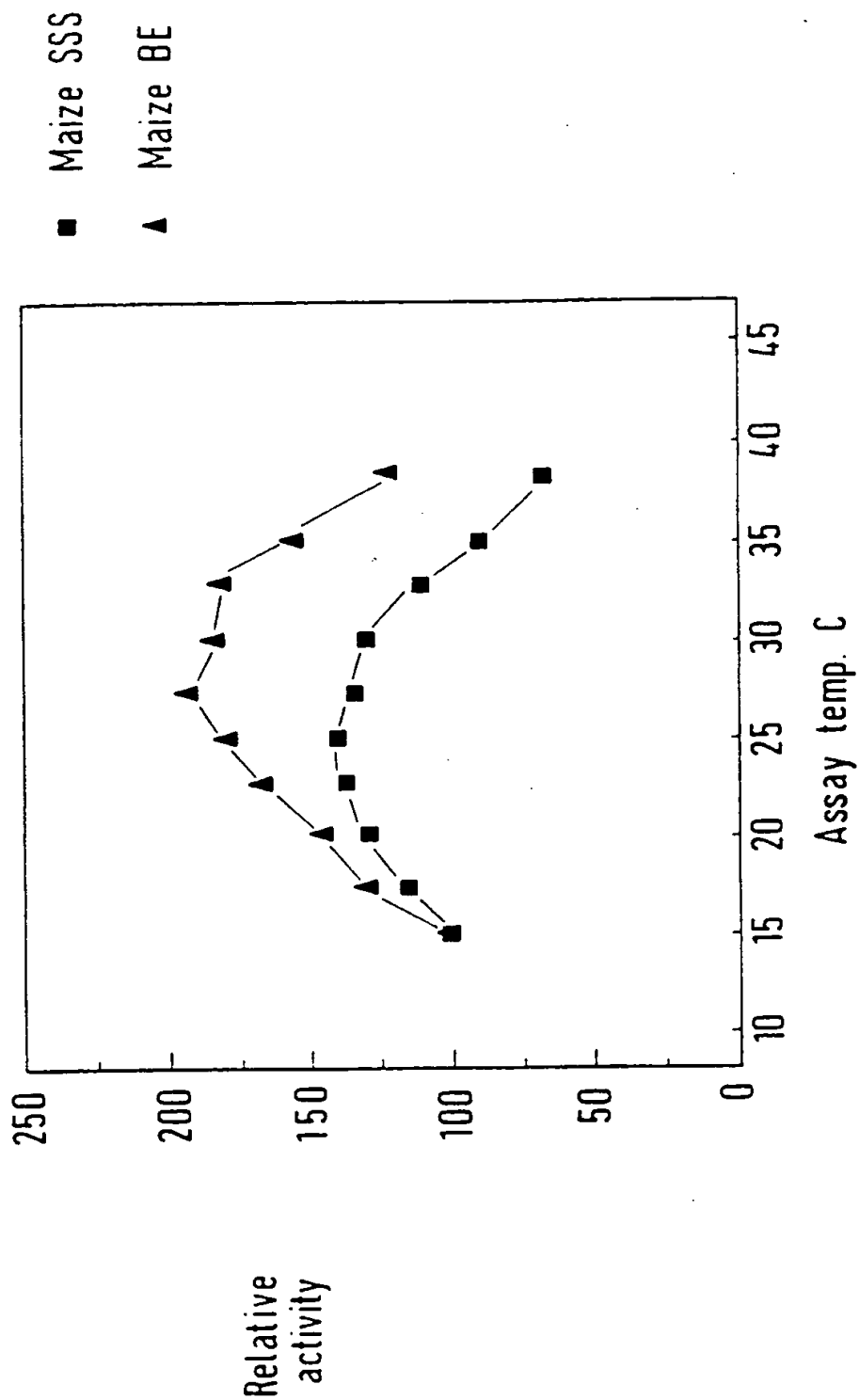


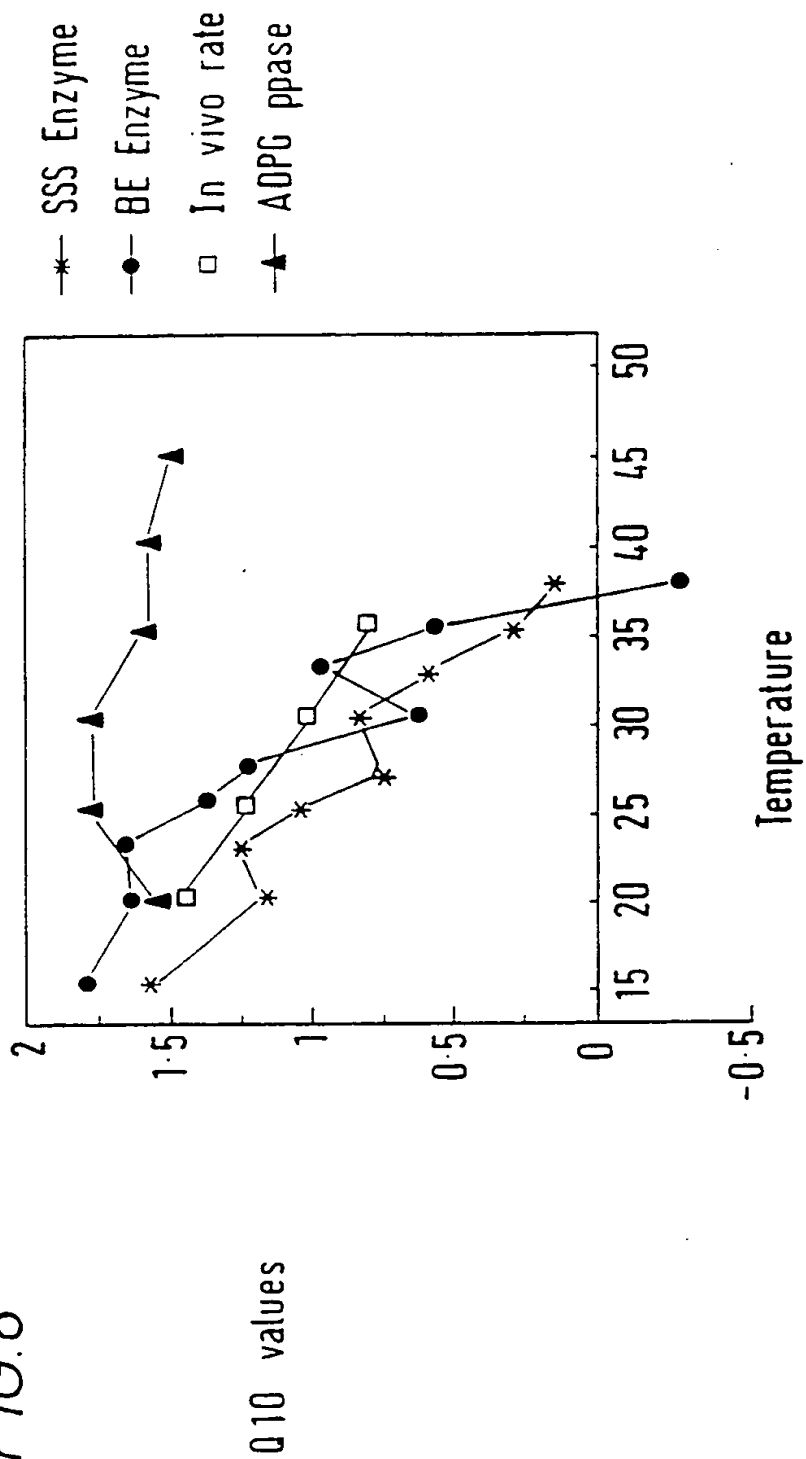
FIG. 7

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8/27

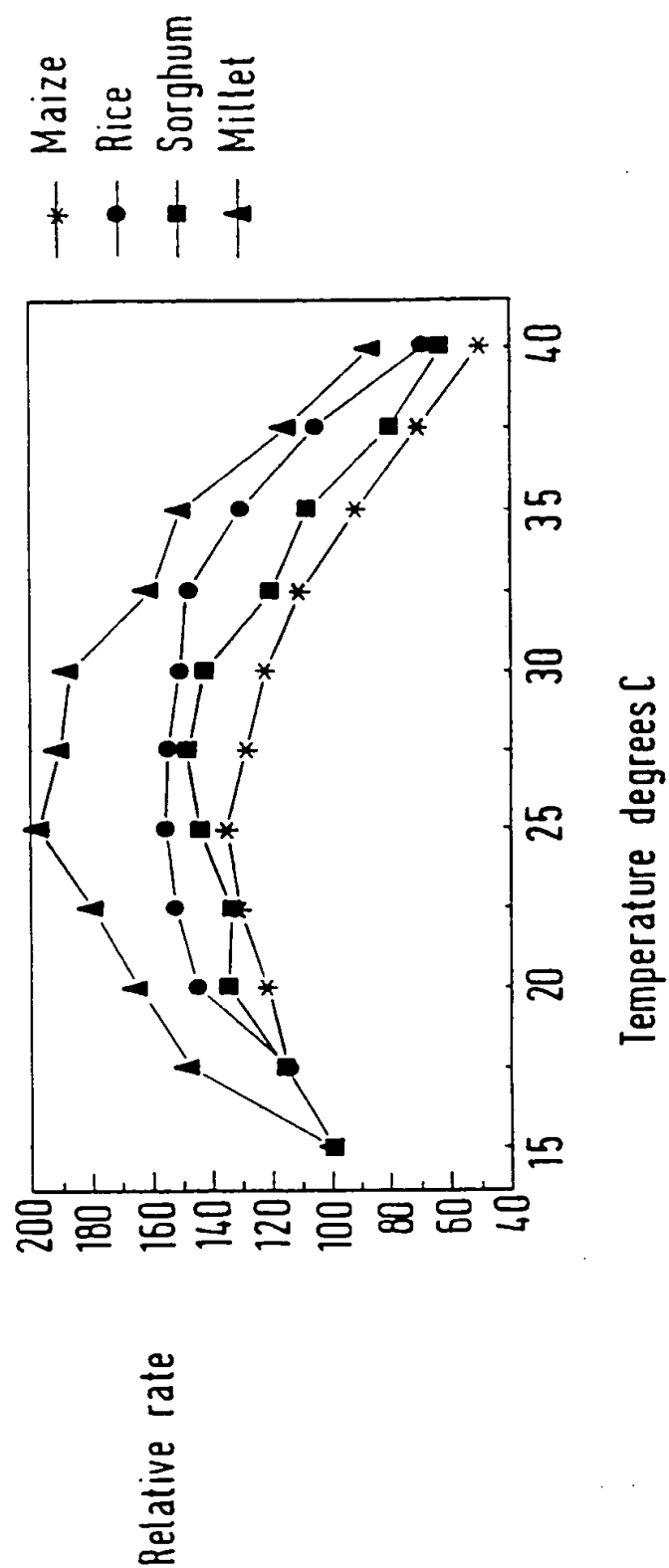
Q10 values for branching enzyme and  
soluble starch synthase

FIG. 8

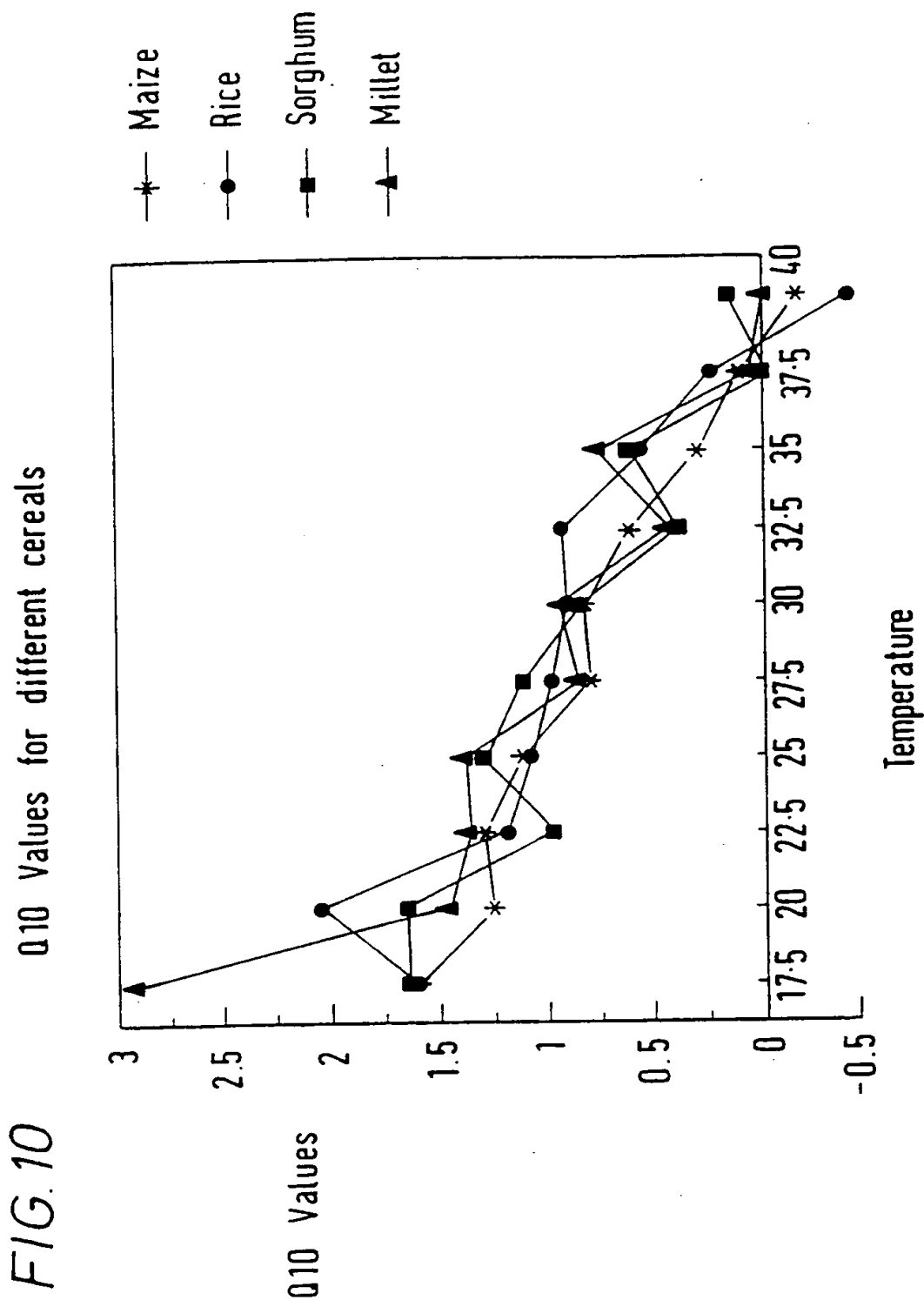


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FIG. 9

SSS Activities from rice, maize,  
sorghum and millet.

10/27

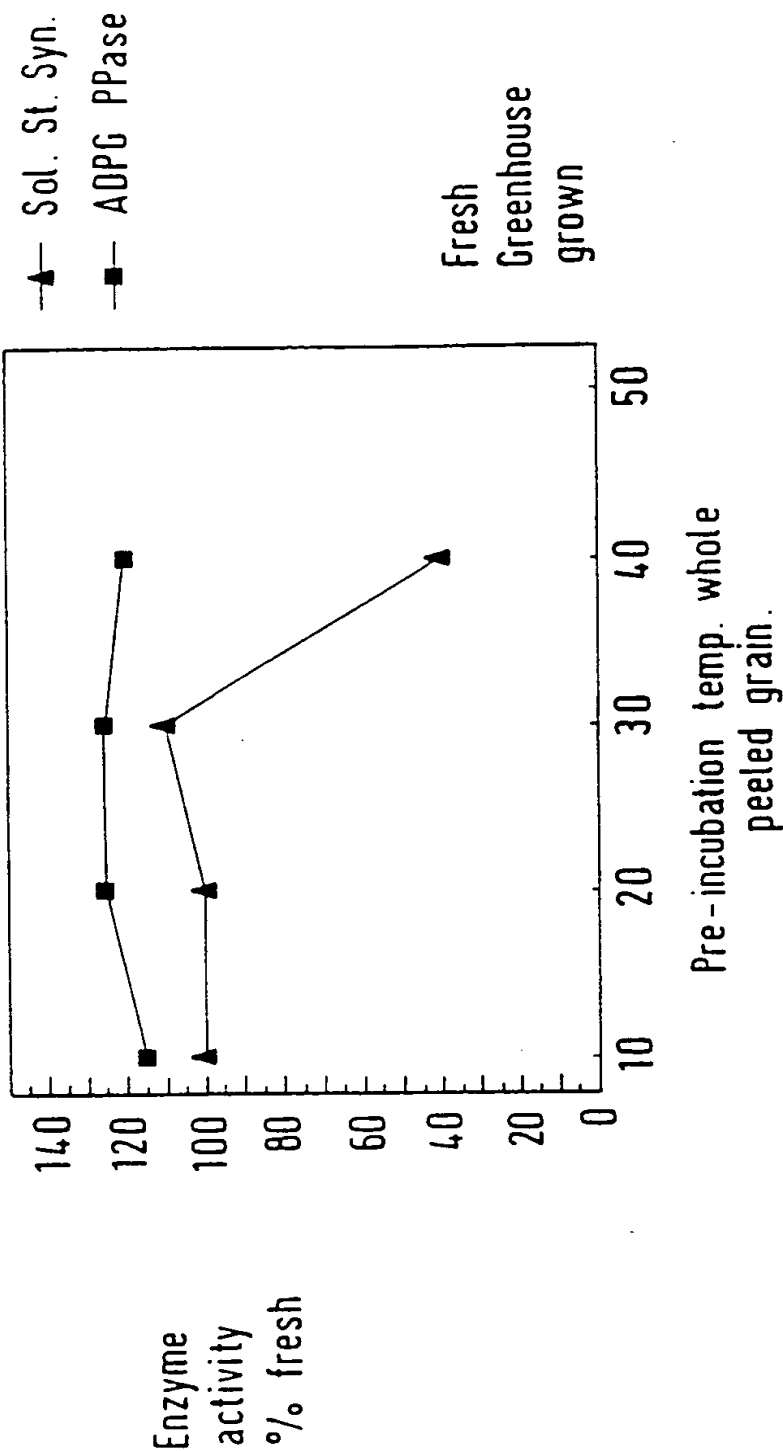


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11/27

Effect of pre-incubation temp. on maize  
SSS assayed at 20 C.

FIG. 11



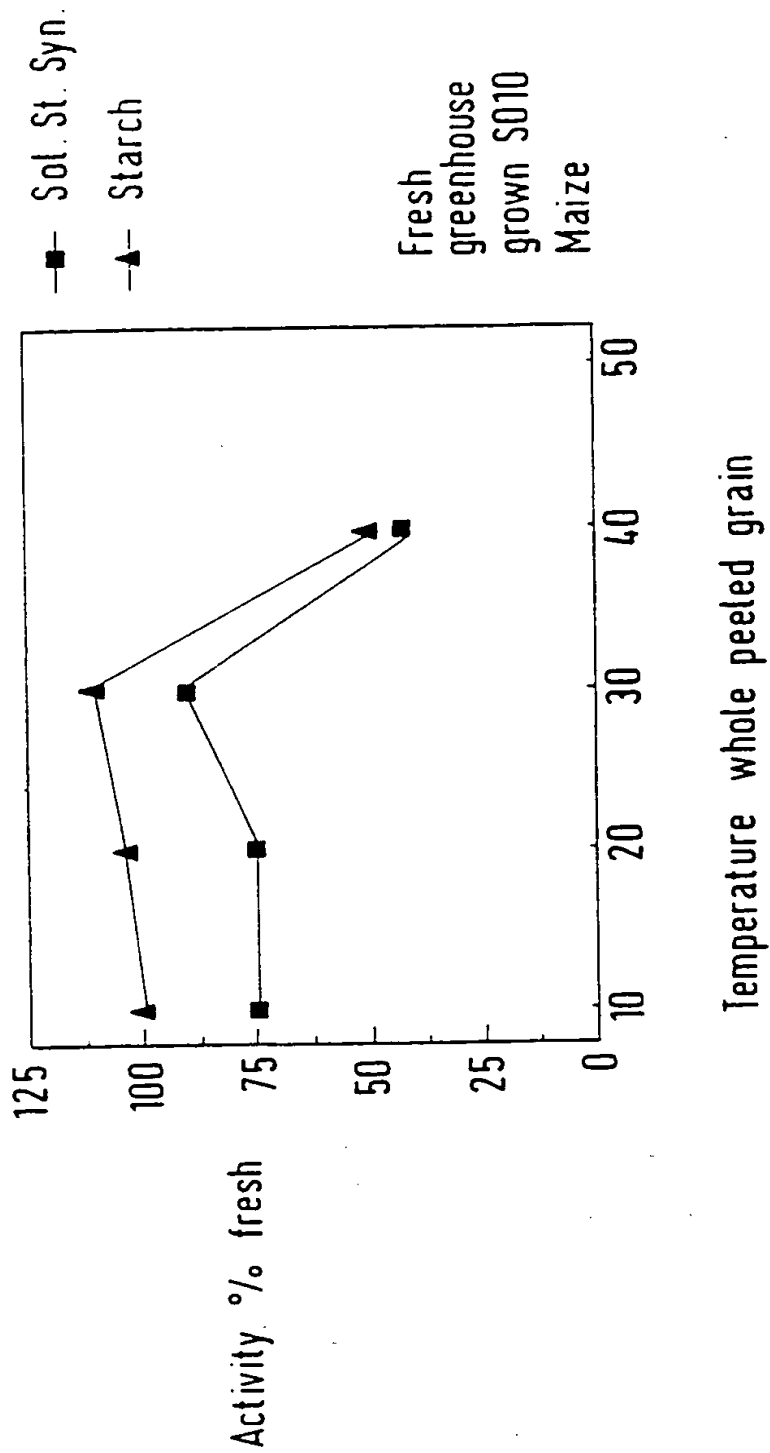
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12/27

SSS activity following pre-incubation temp.  
on starch synthesis and SSS.

FIG. 12

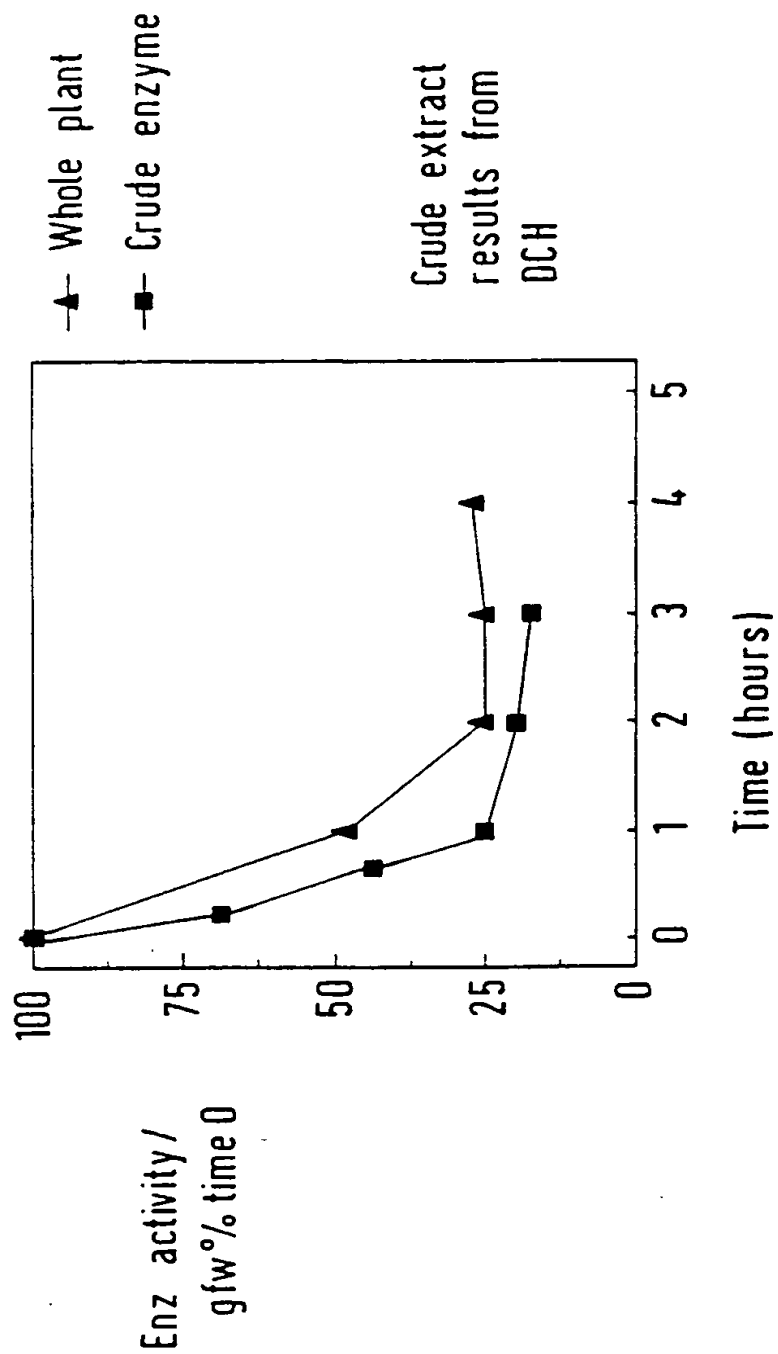


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13/27

SSS activity following pre-inc at 40C in vivo  
and in vitro.

FIG. 13

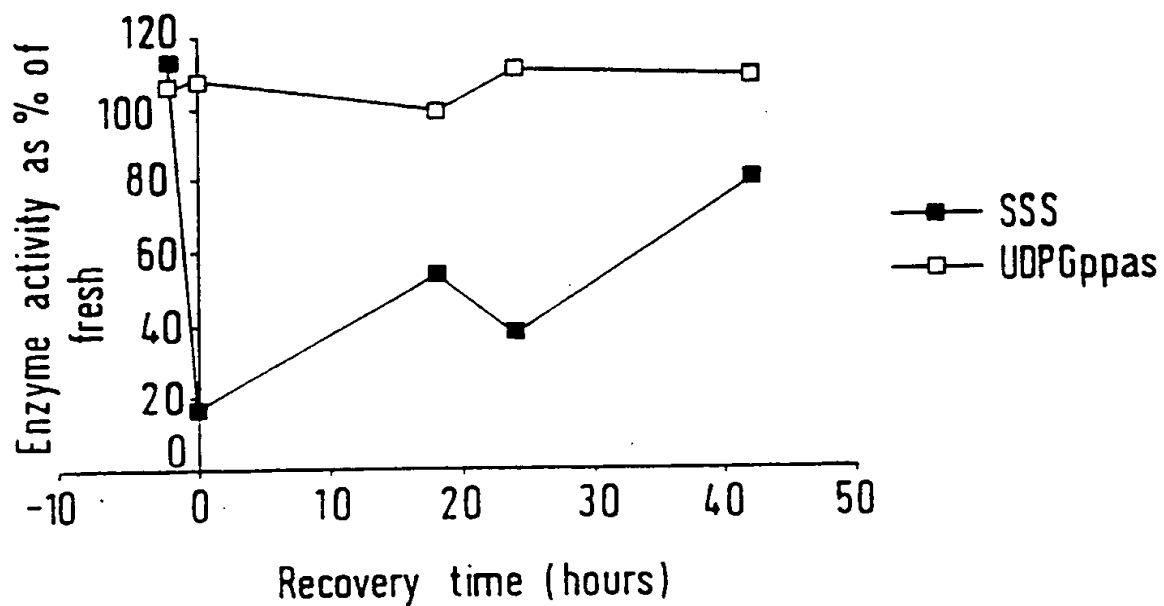


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14/27

FIG. 14

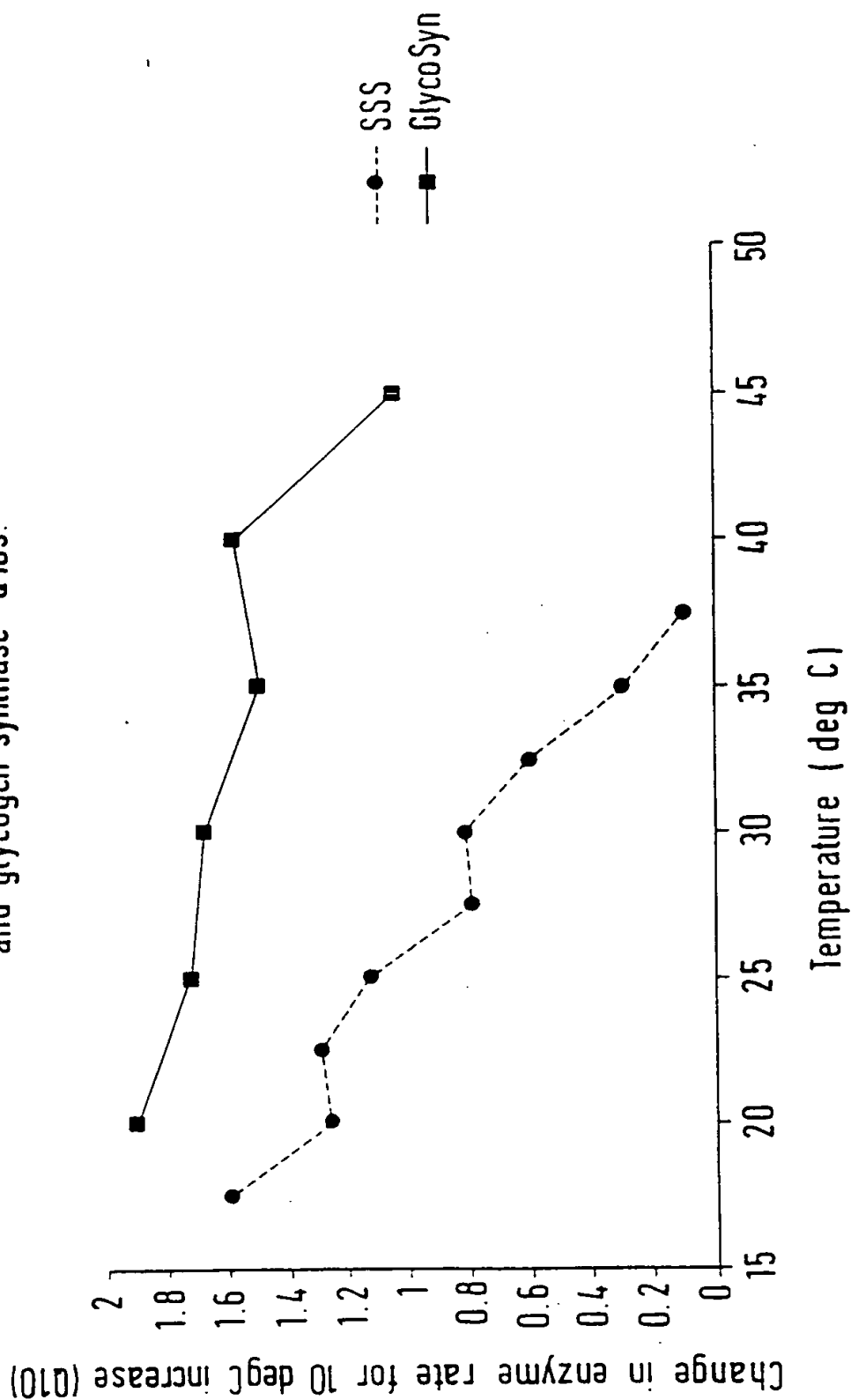
Recovery of SSS and UDPG pyrophosphorylase activity following 37 deg C heat treatment of maize ear.



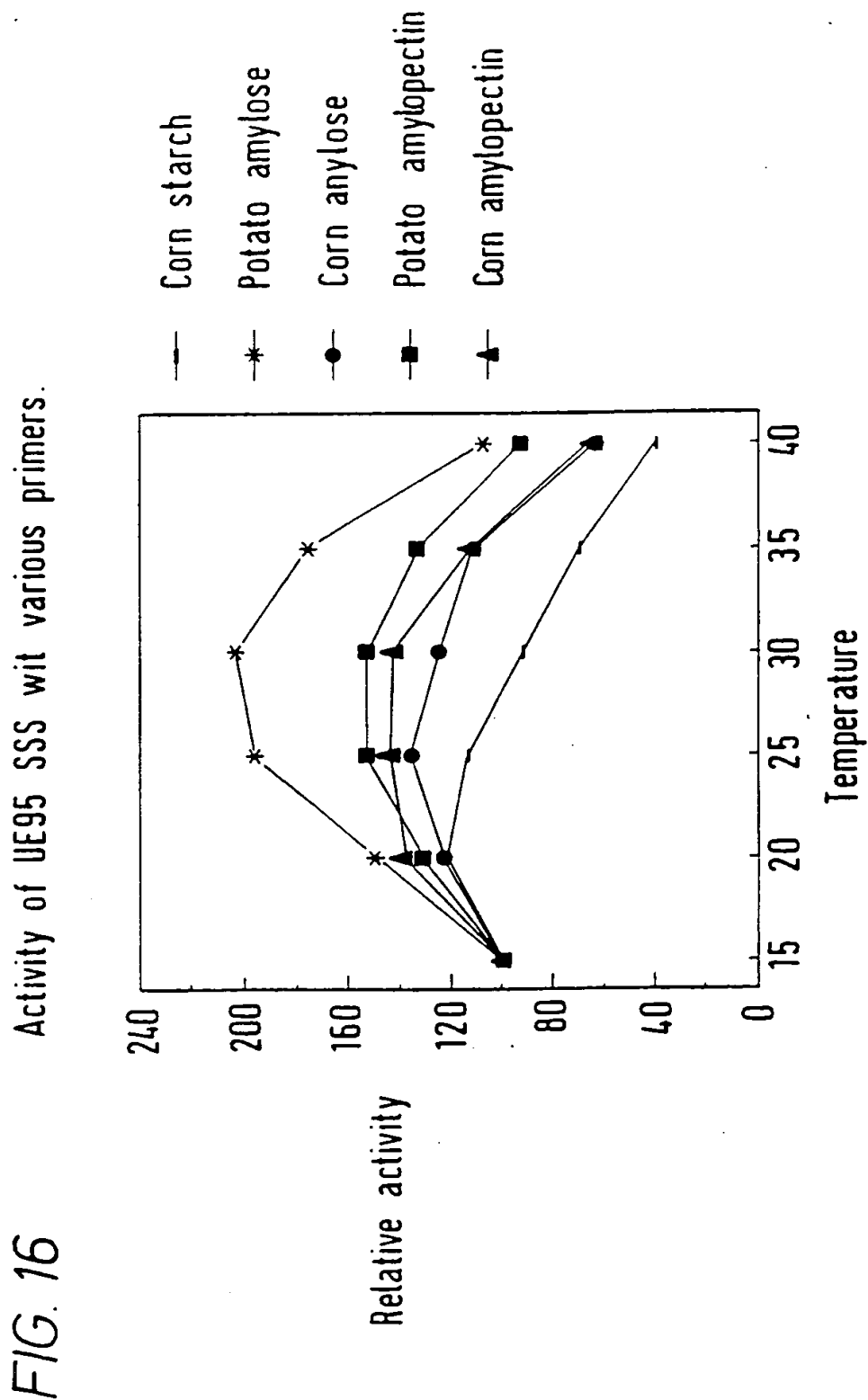
SUBSTITUTE SHEET

15/27

FIG. 15 Effect of temperature on soluble starch synthase and glycogen synthase Q10s.

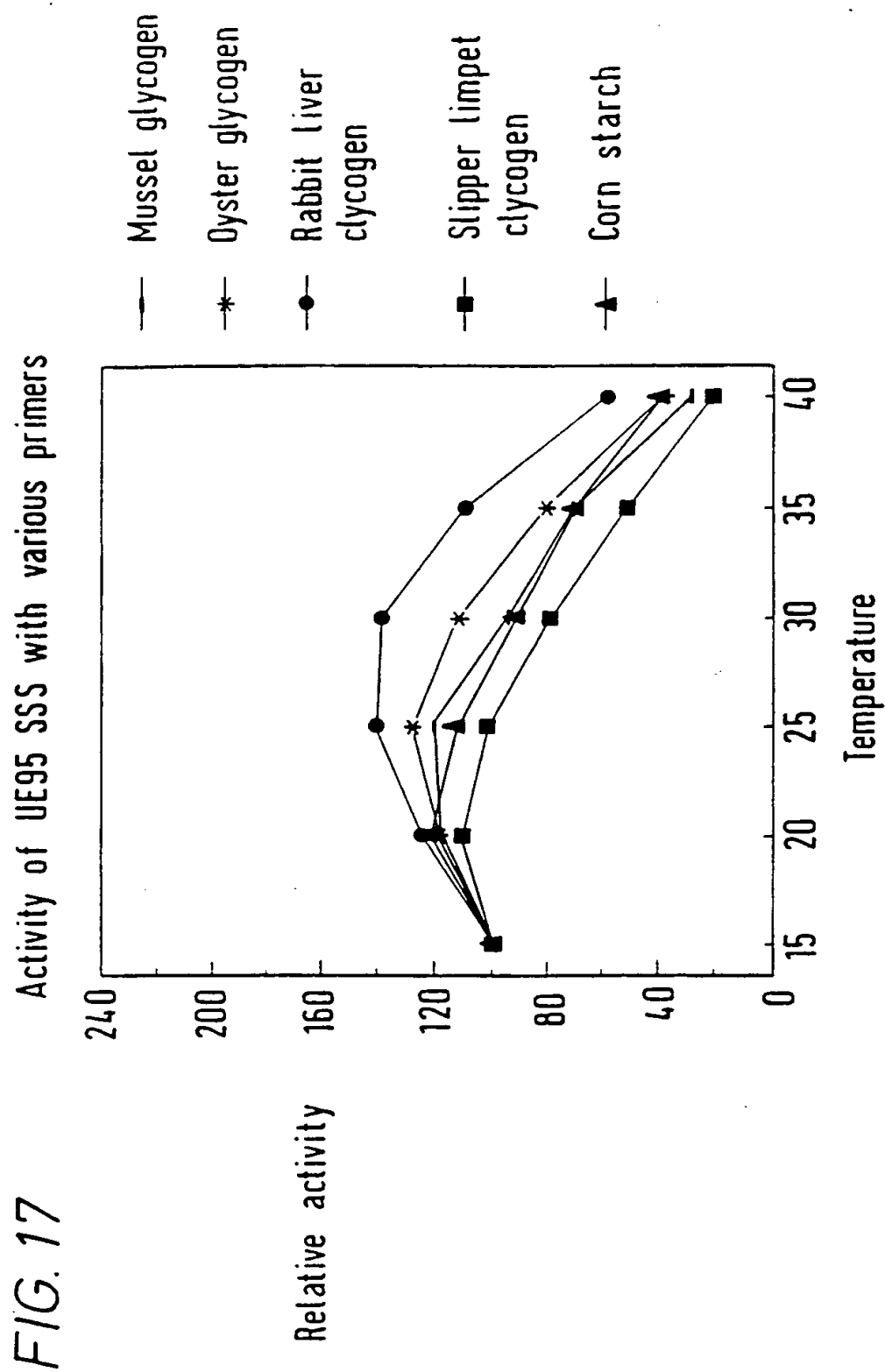


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17/27



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## FIG. 18 (1/3)

ECGLGA	standard; DNA; PRO; 1601 BP
ID	
XX	
AC	J02616;
XX	
DT	19-APR-1990 (Rel. 23; Last updated; Version 1)
DT	29-NOV-1987 (Rel. 14; Created)
XX	
DE	E.coli glgA gene encoding glycogen synthase, complete cds; glgC;
DE	gene encoding glucose-1-phosphate adenylyl transferase, 5' end.
XX	
KW	adenylyltransferase;
KW	ADP-glucose:alpha-1,4-glucan, 4-glucosyltransferase; glgA gene;
KW	glgC gene; glucose-1-phosphate adenylyl transferase;
KW	glucosyltransferase; glycogen synthetase; synthetase.
XX	
OS	Escherichia coli
OC	Prokaryota; Bacteria; Gracilicutes; Scotobacteria,
OC	Facultatively anaerobic rods; Enterobacteriaceae; Escherichia.
XX	
RN	(1)

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## FIG. 18 (2/3)

; RB 1-1601  
 ; RA Kumar A., Larsen C.E., Preiss J.;  
 ; RT "Biosynthesis of bacterial glycogen: Primary structure of  
 ; RT Escherichia coli ADP-glucose:alpha-1,4-glucan,  
 ; RT 4-glucosyltransferase as deduced from the nucleotide sequence of  
 ; RT the glgA gene";  
 ; RL J. Biol. Chem. 261:16356-16259(1986).  
 ; XX  
 ; DR SWISS-PROT; p08323; GLGA\$ECOLI.  
 ; XX  
 ; CC A potential ribosome binding site is located at positions  
 ; CC 1552-1556.  
 ; XX  
 ; FH Key  
 ; FH Location/Qualifiers  
 ; FT CDS  
 ; FT 114..1547  
 ; FT /note="glcogrn synthase (EC 2.4.1.21)  
 ; FT /nomgen="glgA"  
 ; FT 1566..>1601  
 ; FT CDS  
 ; FT /note="glucose-1-phosphate adenylytransferase  
 ; FT (EC 2.7.7.27) /nomgen="glgC"  
 ; XX  
 ; SQ Sequence 1601 BP; 331 A; 405 C; 467 G; 398 T; 0 other;  
 ; CC Retrived by M216378 on Mon 18 Nov 91 8:43:12-PDT using Findseq  
 ECGLGA



FIG. 18 (3/3)

gtgattggtgaaaacgcagaggaagatgcacgtcgtttctatcgttcagaagaaggcatcgtgctggtaa  
cgcgcaaatgctacggaagttagggcataaacaggagcgataatgcagggttttacatgtatgttgagag  
atgtcccgcgtgcttaaaacggcggtctggctgagtatttggggcattaccgcgacacaaatcgag  
acggcgttgacgctcggtactgttgccctgcatttcccgacattcgccgtggcgtgacgatgcgcagg  
agtatcccgtcgtgataacctccggacatatcacgctgttgttcggctattacaacggggttggcatt  
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ataccgacaacgtattgcgttttgcgtgctggggtgggttggggcagaaatggccagcgggcttgaccc  
attcgggcttcctgatgtggtgcacacgactggcatgcaggccttgcgctgcgtatctggcggcg  
cgggcggtccggcgaagtctgtttactgtgcacaacctagcctatcaaggcatgttttatgcacatc  
acatgaatgacatccaattgccatggtcattctttaatatcatgggctgggaattcaacggacaatatc  
ttcctgaaggccggtctgtactatgccgatcacattacggcggtcagtcacaacctacgttcgcgagatc  
accgaaccgcagtttgcctacggtatggaaaggtctgttgcaacagcgtcacgcgaaaggcgtctttccg  
gcgtaccgaacggcgtggacgagaaaatctggagtcagagacggacttactgttggcctcgcgttacac  
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cctcaccgggttcttcggagcagggcgggcagctggcgctactcggcgcgggcgatccggctgcaggga  
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tcgcatcgcatatatggcgggcgggacttcattatggtgcccagccgtttcgaaccgtcgggcttaacgc  
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tcgctgttacggactattcgacgtgcttttgtactgtggtcctgtcctccactgtggcggtttgtgcaac  
gtcaggctatggcaatggattttggctggcagggtcgggcgaagtcgtaccgtgagctttactatcgctc  
gaaatagttttcagggaacgccctacatgaatgctccgtttacatatcgcgccacgctt

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# Construction of Glycogen Synthase Vectors

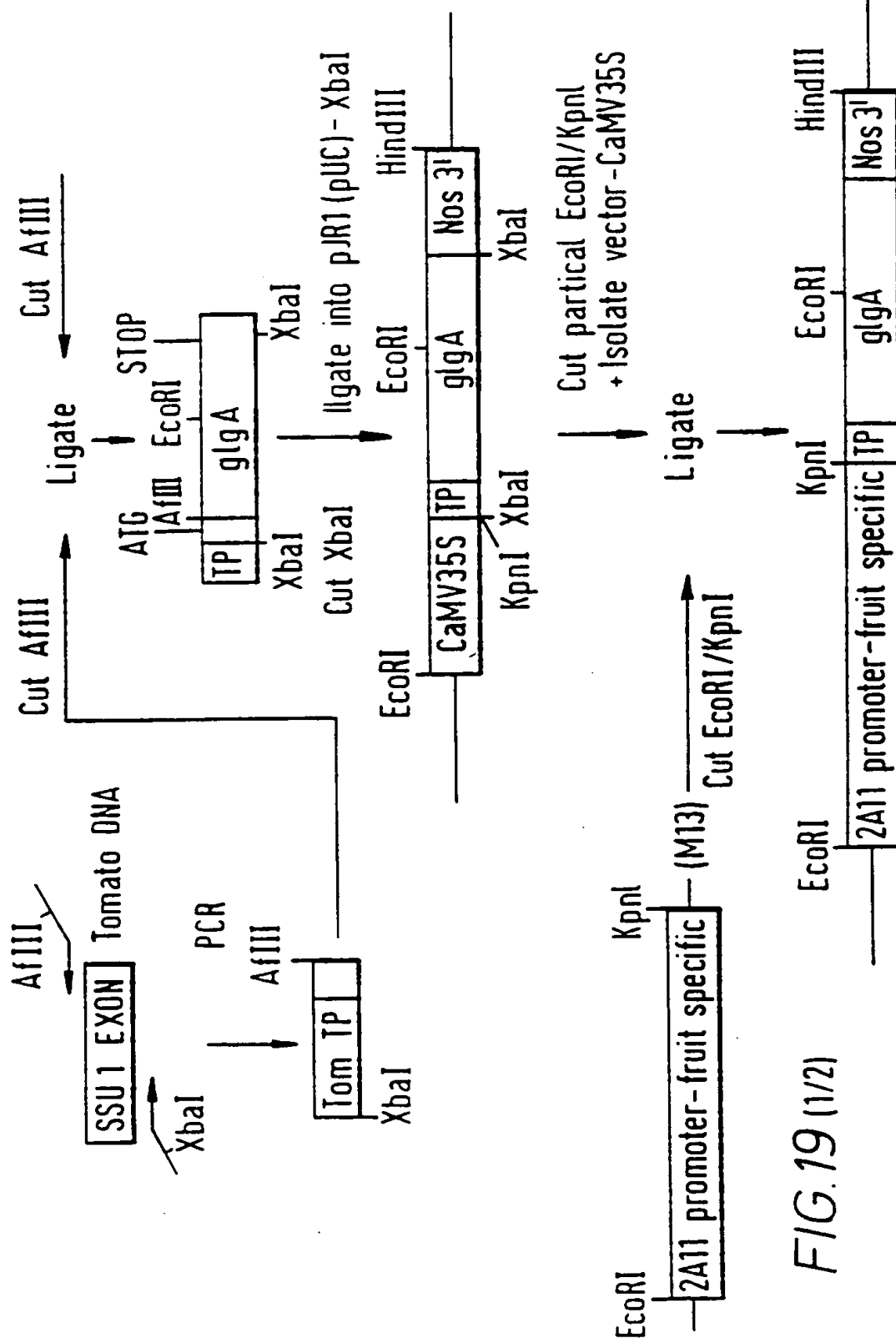
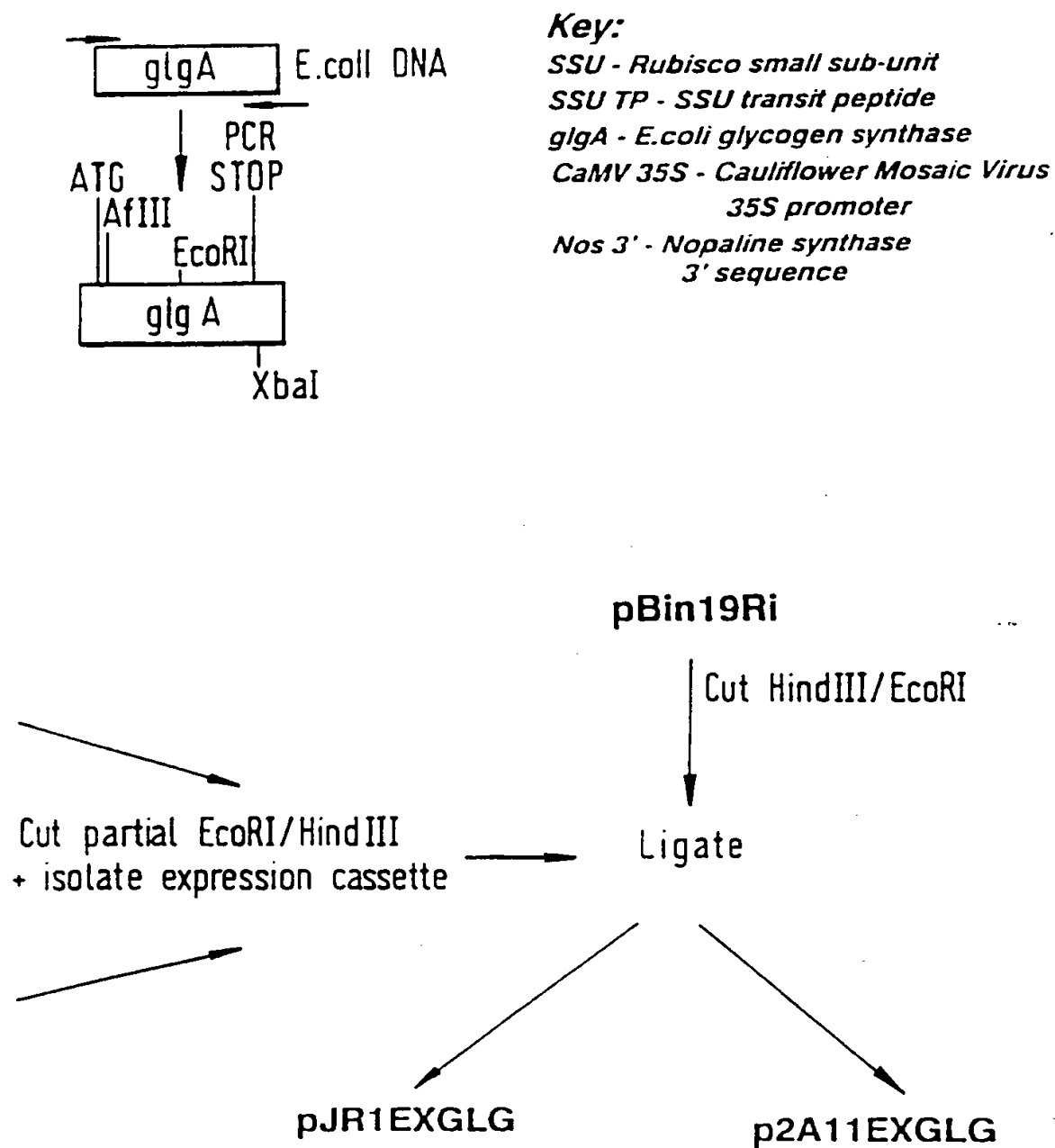


FIG.19 (1/2)

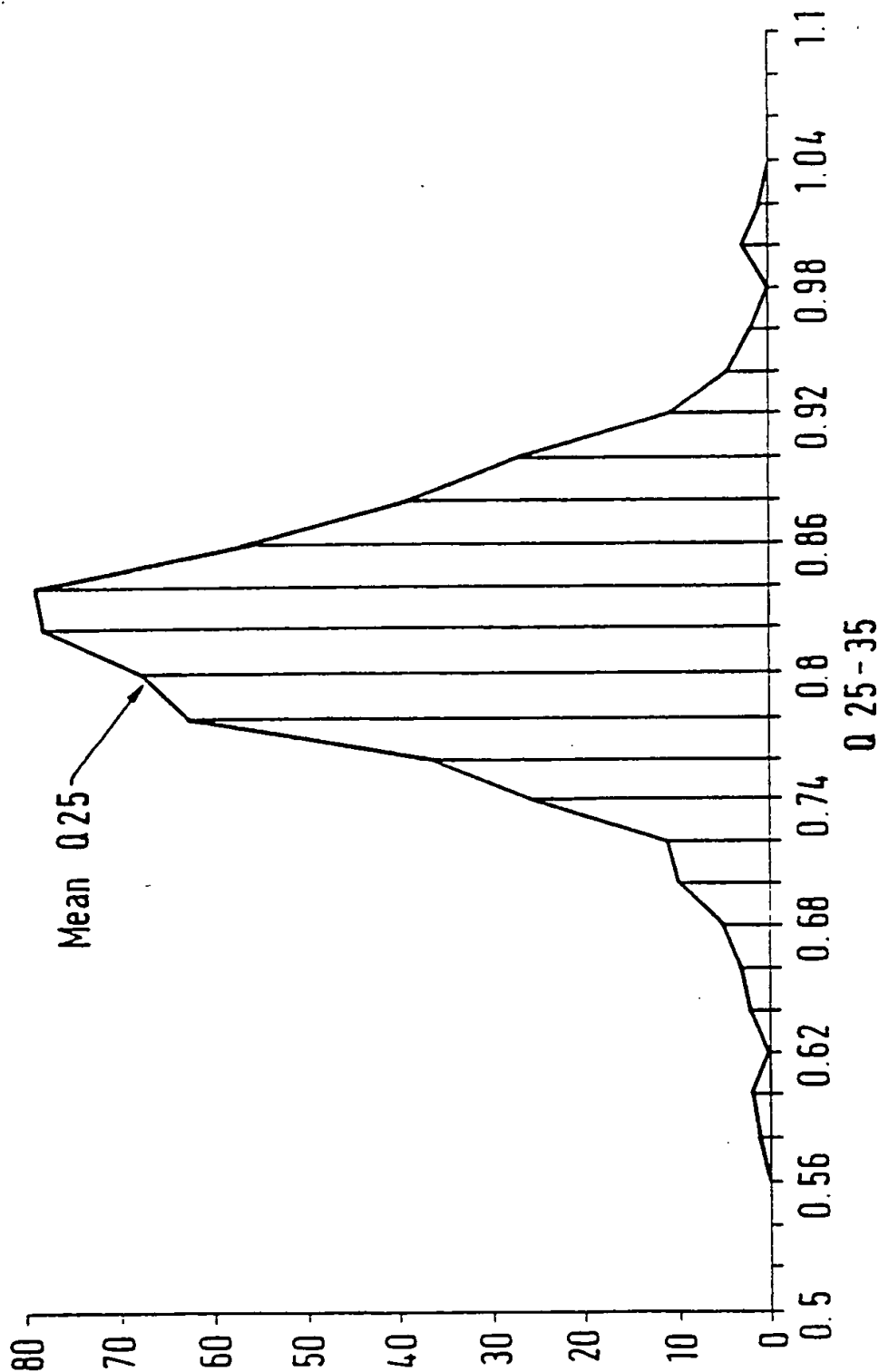
22/27

FIG. 19 (2/2)



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FIG. 20 Frequency distribution for Q25-35



Effect of temperature on relative activity  
of SSS from various genotypes versus UE95.

FIG. 21

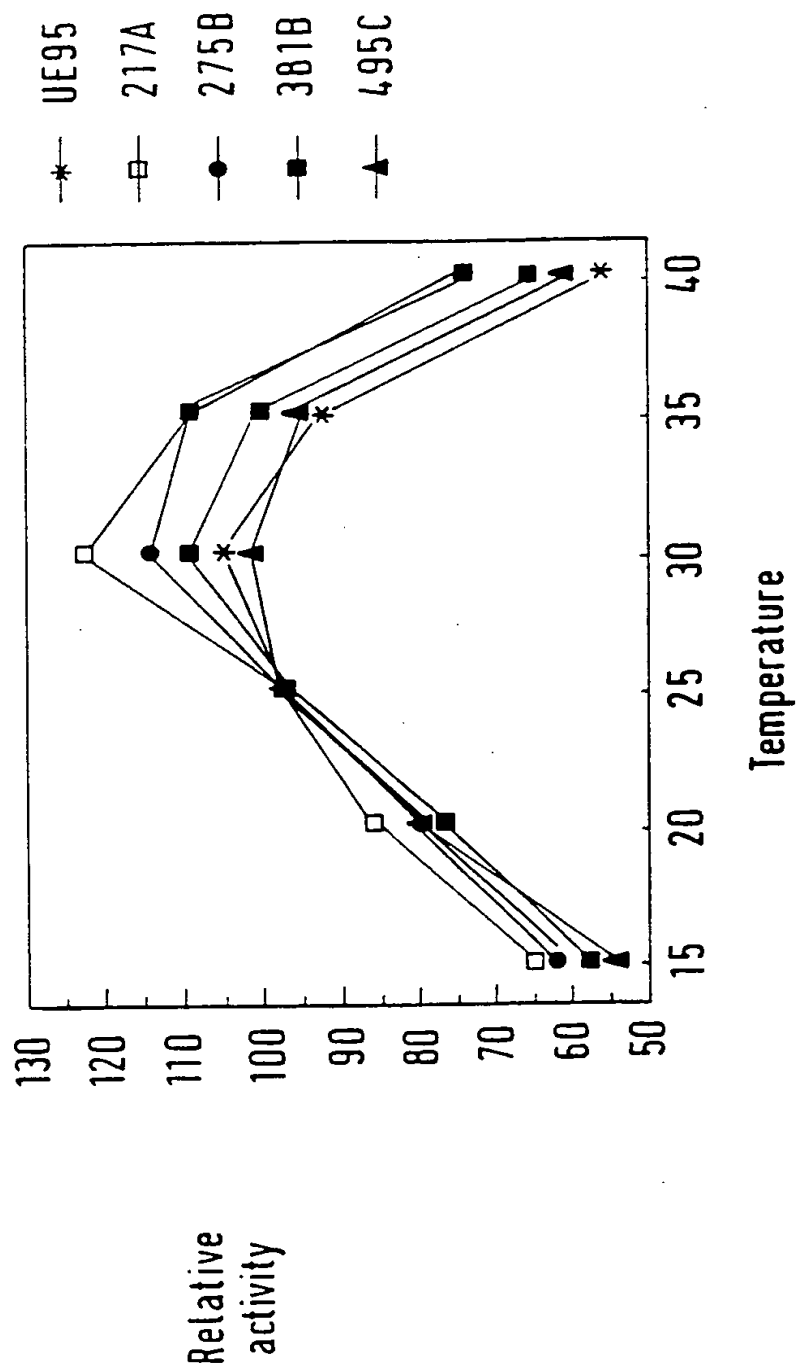
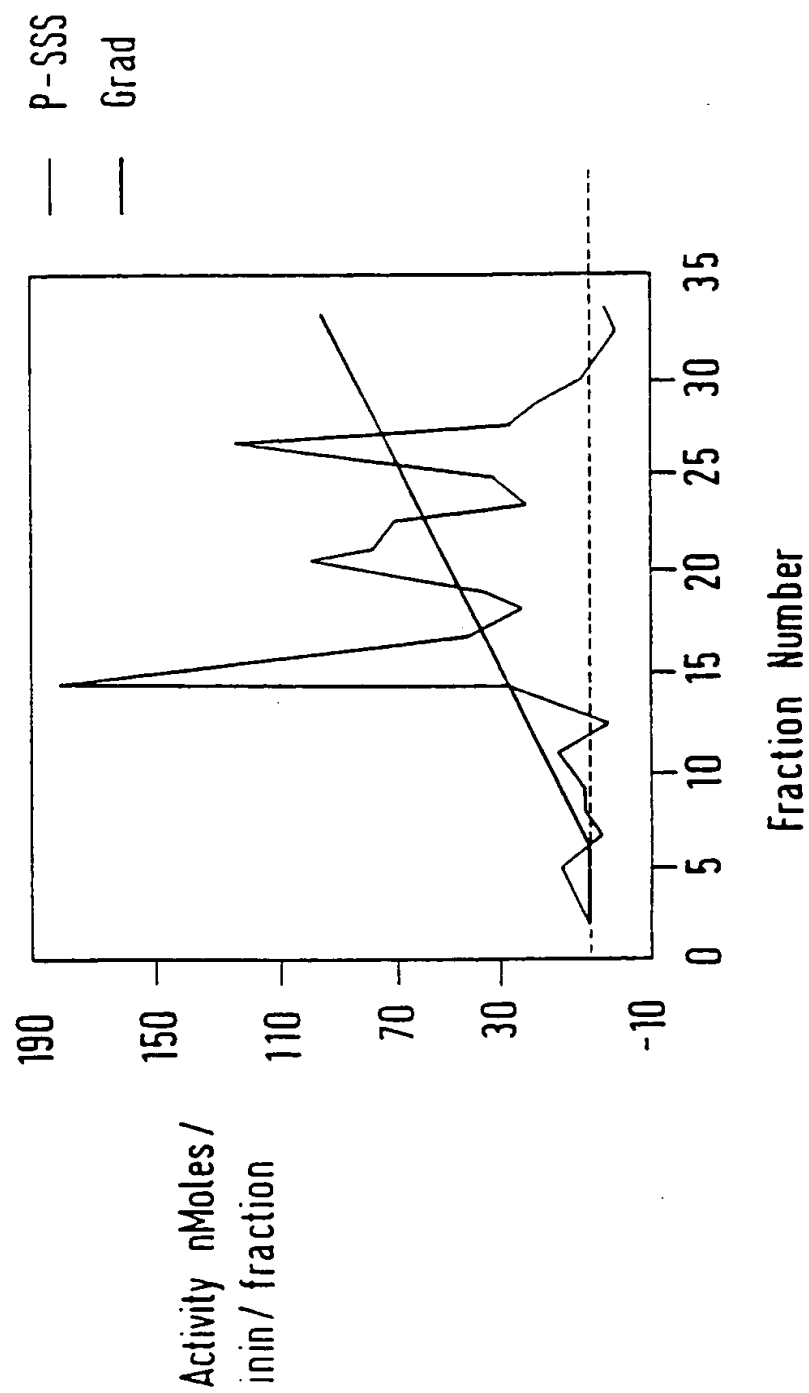


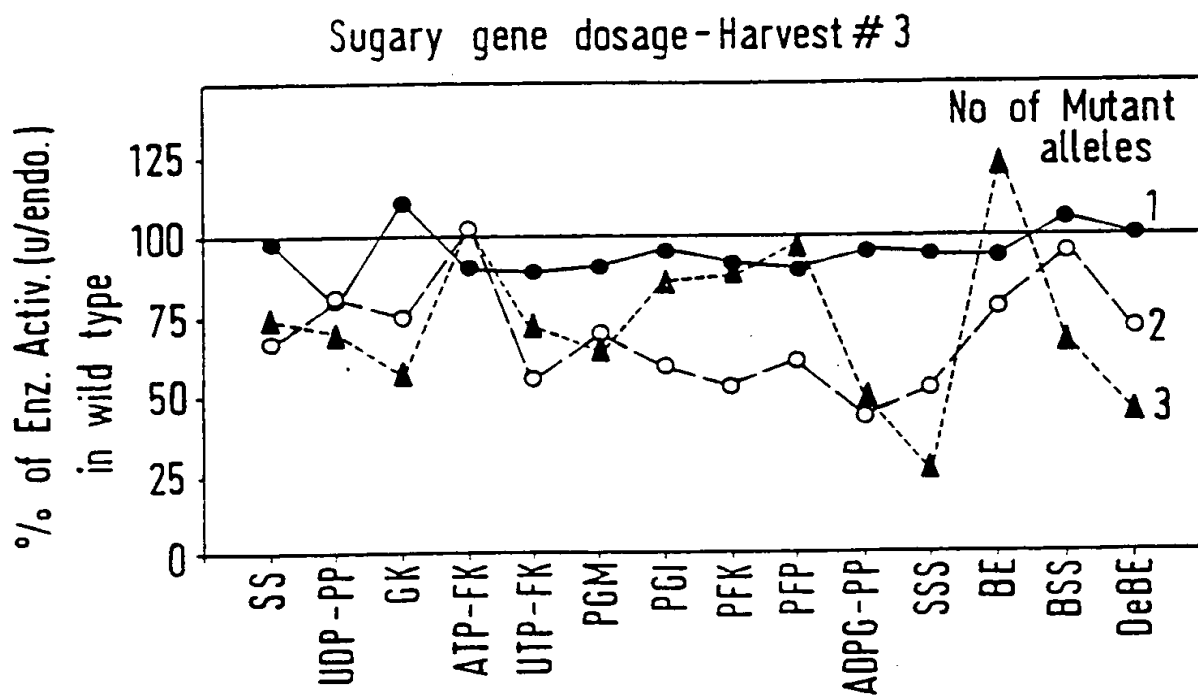
FIG. 22

Elution of soluble starch synthase  
activity from Mono-Q Column



26/27

FIG. 23



SUBSTITUTE SHEET

FIG. 24

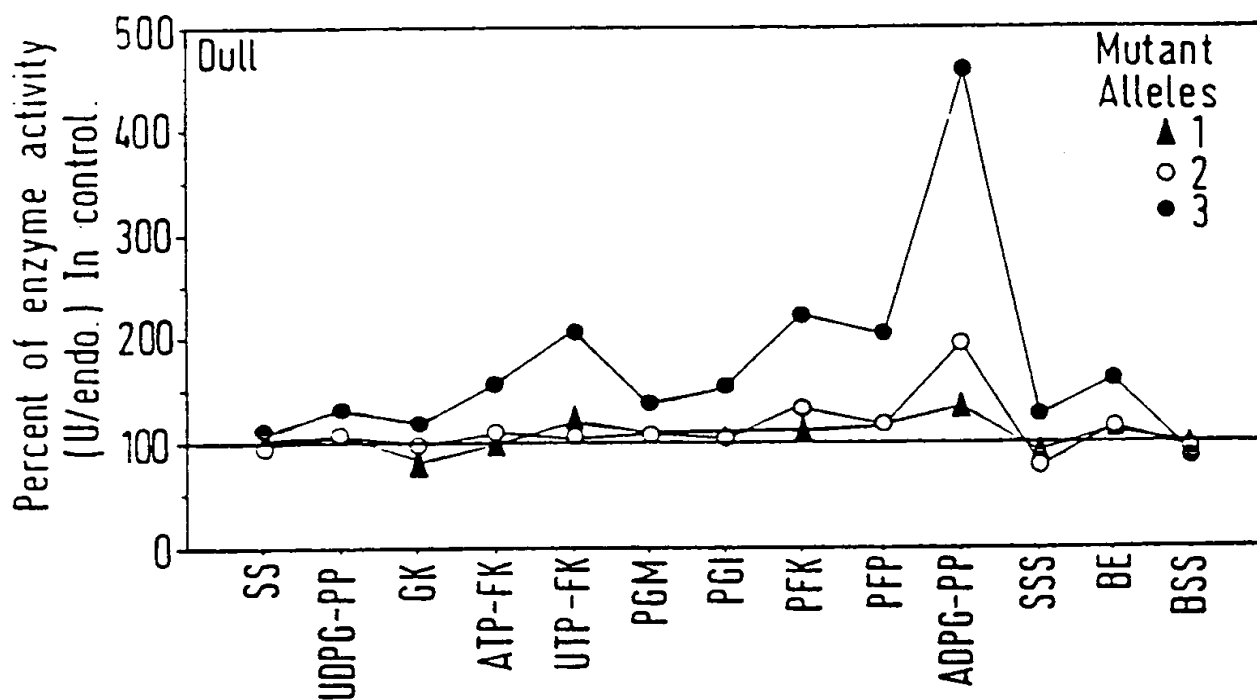
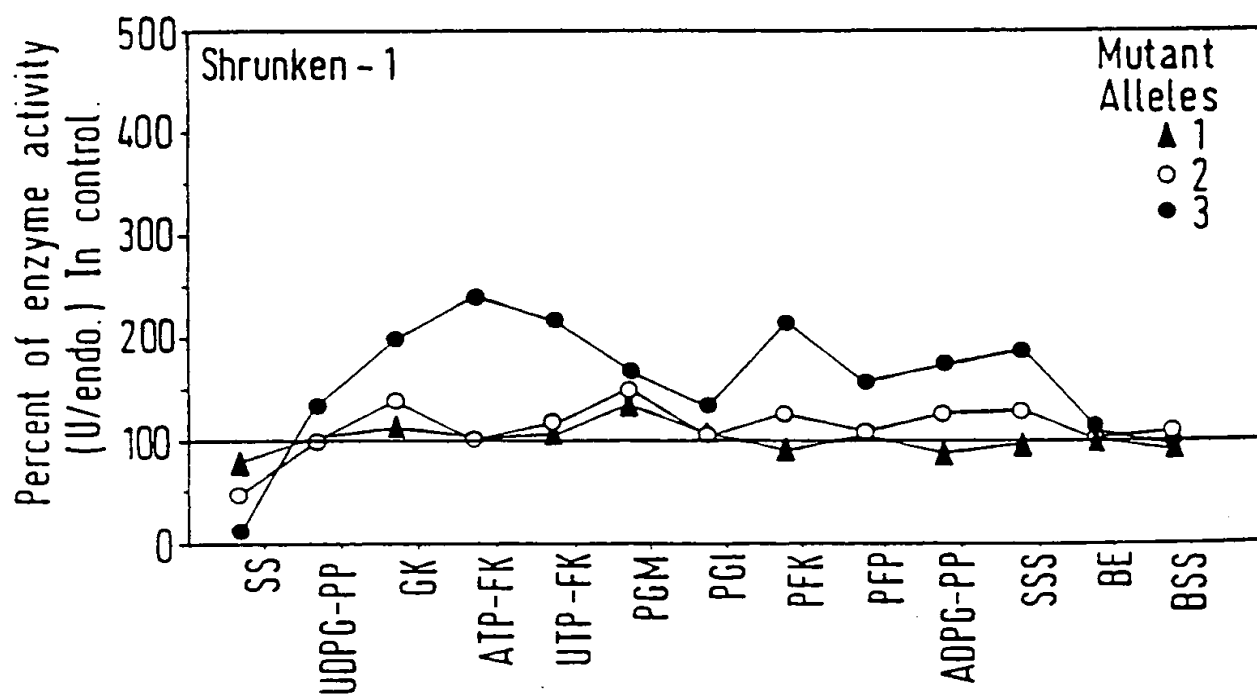


FIG. 25





## INTERNATIONAL SEARCH REPORT

PCT/GB 92/01881

International Application No

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/82;	C12N15/54;	A01H1/00; A01H5/00
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; A01H	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO,A,9 211 382 (CALGENE) 9 July 1992  see page 42, line 30 - page 46, line 10 ---	1-4,8, 11,15, 19-22, 26,29,39
X	WO,A,9 214 827 (INSTITUT FUR GENBIOLOGISCHE FORSCHUNG BERLIN) 3 September 1992  see the whole document --- -/-	1,3,5, 11,13, 15-17, 19,21, 23,29, 31,38,39
<p><sup>10</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 28 JUNE 1993		Date of Mailing of this International Search Report 11 JUL 1993
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

## INTERNATIONAL SEARCH REPORT

PCT/GB 92/01881

International Application No

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	WO,A,9 119 806 (MONSANTO) 26 December 1991  see the whole document ---	1,2,8, 12,15, 18-20, 26,30, 34,38,39
X	EP,A,0 372 358 (UNIV. IOWA. RES. FOUND.) 13 June 1990  see the whole document ---	1,3,6, 11,14,16, 17,19, 21,23, 24,29, 32,33
X	PLANT PHYSIOLOGY. SUPPL. vol. 93, no. 1, May 1990, ROCKVILLE, MD, USA. page 24 SINGLETARY, G.W., ET AL. 'Sugar metabolism in endosperm of gene dose series involving starch mutants of maize' see abstract 133 ---	1,3,5,6, 11,14, 16,17, 19,21, 23,24, 29,32,33
X	ABSTRACTS VIITH INTERNATIONAL CONGRESS ON PLANT TISSUE AND CELL CULTURE.AMSTERDAM JUNE24-29, 1990. page 177 VAN DER LEIJ, F.R., ET AL. 'Expression of the gene encoding granule bound starch synthase after introduction in an amylose-free and a wildtype potato (Solanum tuberosum)' see abstract A5-28 ---	1,3,5, 19,21,23
X	MOLECULAR AND GENERAL GENETICS vol. 225, 1991, BERLIN DE pages 289 - 296 VISSER, R.G.F., ET AL. 'Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs' see the whole document ---	1,3,13, 15,19, 21,23, 31,39

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## INTERNATIONAL SEARCH REPORT

PCT/GB 92/01881

International Application No

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	<p>BIOLOGICAL ABSTRACTS vol. 89  , 1990, Philadelphia, PA, US;  abstract no. 48351,  DANG, P.L., ET AL. 'Comparison of soluble  starch synthases and branching enzymes  from leaves and kernels of normal and  amylose-extender maize'  see abstract  &amp; BIOCHEM. GENET.  vol. 27, no. 9/10, 1989,  pages 521 - 532</p> <p>---</p>	<p>1,3,5,6,  11,14,  16,17,  19,21,  23,24,  29,32,33</p> <p>38-40</p>
A	<p>PLANT PHYSIOLOGY.  vol. 99, no. 1, May 1992, ROCKVILLE, MD,  USA.  page 87  FISHER, D.K., ET AL. 'Cloning and  characterization of cDNAs for starch  branching enzyme in Zea mays L.'  see abstract 519</p> <p>---</p>	<p>6,11</p>
A	<p>PLANT PHYSIOLOGY. SUPPL.  vol. 99, no. 1, May 1992, ROCKVILLE, MD,  USA.  page 22  MA, S., ET AL. 'Soluble starch synthase  from sweet corn'  see abstract 131</p> <p>---</p>	<p>1,11</p>
E	<p>WO,A,9 309 237 (SANDOZ)  13 May 1993</p> <p>see the whole document</p> <p>-----</p>	<p>1,5,6,  13,  15-17,  19,23,  24,31-33</p>

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9201881  
SA 65439

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
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EP-A-0372358	13-06-90	US-A- 5004864 AU-B- 625542 AU-A- 4561689	02-04-91 16-07-92 31-05-90
WO-A-9309237	13-05-93	None	

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82